Characterization of the biochemistry and physiology of hydrocarbon degradation pathways by stable isotope approaches

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GLOSSARY

AKIE	Apparent kinetic isotope effect
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BTEX	Benzene, toluene, ethylbenzene, xylene
CoA	Coenzyme A
Cr/HTC	Chromium-based High-Temperature Conversion
CSIA	Compound specific isotope analysis
DIN	Deutsches Institut für Normung
DNA	Deoxyribonucleic acid
DNAPL	Dense non-aqueous phase lipids
DOC	Dissolved oxygen concentration
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EA	Elemental analyzer
EbDH	Ethylbenzene-dehydrogenase
FAD	Flavin adenine dinucleotide
FID	Flame ionization detector
GC	Gas chromatography
HPLC	High performance liquid chromatography
IRMS	Isotope ratio mass spectrometry
HS-SPME	Headspace-solid phase microextraction
HS-PRV	Headspace-programmed temperature vaporizer
LC-IRMS	Liquid chromatography-isotope ratio mass spectrometry
KIE	Kinetic isotope effect
LNAPL	Light non-aqueous phase lipids
MDL	Method detection limit
MMI	Multimode Inlet
MTBE	Methyl tert-butyl ether
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NA	Natural attenuation
NADH	Nicotinamide adenine dinucleotide
NCIMB	National collections of industrial, marine and food bacteria
OD_{578}	Optical density measured at a wavelength of 578 nm
OTU	Operational taxonomic units
PCE	Tetrachloroethylene
PCMH	*p*-Cresol methyl hydroxylase
PCR	Polymerase chain reaction
pH	*Potentia hydrogenii*
PTFE	Polytetrafluoroethylene
PTV	Programmed temperature vaporizer
rRNA	Ribosomal ribonucleic acid
SIP	Stable isotope probing
SPME	Solid phase microextraction

TAM	*Thauera aromatic* medium
Taq	*Thermus aquaticus*
TCE	Trichloroethylene
TE	Tris-EDTA
TEX	Toluene, ethylbenzene, xylene
TFAA	Trifluoroacetic anhydride
TM	Tris HCl- magnesium sulfate
Tris	Tris-hydroxymethyl-aminomethane
UFZ	Umweltforschungszentrum
UPLC	Ultra-performance liquid chromatography
UV	Ultraviolet
V-PDB	Vienna-Pee Dee-Belemnite

SUMMARY

Biodegradation plays an important role in the removal of hydrocarbons from the environment. Compared to the extensive studies concerning aerobic hydrocarbon degradation, there is still a lack of knowledge with regard to the degradation of hydrocarbons under anoxic conditions. The current thesis, containing three subprojects, focused on the anaerobic degradation of the BTEX (abbreviation for benzene, toluene, ethylbenzene, xylene) compound - especially ethylbenzene, *n*-alkanes and phenols. Phenols are close relatives of BTEX compounds; the biodegradation pathways of phenols and BTEX share many similarities with regard to the activation mechanisms and the enzymes involved. Stable isotope analysis was applied in this thesis as the main tool for the characterization of hydrocarbon degradation pathways and involved microorganisms. The carbon or hydrogen stable isotope fractionation patterns upon biodegradation of TEX, *n*-alkanes and phenols were determined to elucidate the degradation pathways. Carbon stable isotope labelling method was applied to identify the key-players in an ethylbenzene-degrading enrichment culture.

The three subprojects are as follows:

i) Characterization of freshwater ethylbenzene-degrading enrichment cultures under sulfate-and nitrate-reducing conditions.
ii) Determination of the activation mechanism of the *n*-hexadecane-degrading strain *Desulfococcus oleovorans* strain Hxd3.
iii) Development of a compound specific isotope analysis (CSIA) based method for the detection and quantification of (alkyl) phenol degradation in the environment.

Ethylbenzene, a petroleum hydrocarbon, member of the BTEX compounds, is frequently detected in groundwater and soil due to improper industrial disposal, spillage and accidents. Ethylbenzene degradation under strictly anoxic conditions has been rarely reported for freshwater environments and the responsible microorganisms and their degradation pathway(s) are currently unknown. In the present thesis, an anaerobic ethylbenzene-degrading culture obtained from coarse sand taken from an on-site column system percolated with benzene contaminated groundwater from an aquifer in Zeitz, Germany, was successfully established. Ethylbenzene degradation was coupled to sulfide production. By using ^{13}C-labeled ethylbenzene as substrate, it was revealed that

ethylbenzene was mineralized to CO_2 by the microbial consortia. The identification of the microorganisms metabolizing ethylbenzene by stable isotope probing approach has failed due to the extremely slow growth of the culture. Metagenomic as well as metaproteomic analysis showed that the phyla Firmicutes, Proteobacteria and Chloroflexi were the dominant groups in the enrichment culture. At the contaminated site Òdena, located close to Barcelona, Spain, compound specific isotope analysis (CSIA) was applied to study TEX biodegradation in a contaminated aquifer near a former chemical plant. By analyzing the carbon isotope composition in the groundwater samples from different sampling wells along the groundwater flow, a biodegradation process of TEX along the groundwater flow was suggested. By the combination of cultivation experiments and 16S rDNA analysis, the nitrate reducing phylotypes belonging to the genera *Azoarcus* and *Thauera* were found to be main degraders of ethylbenzene at this field site by coupling the process to nitrate reduction. Moreover, the CSIA analysis of the ethylbenzene degradation by enrichment cultures containing *Thauera* or *Azoarcus* as dominant phylotypes indicated side chain-hydroxylation is the initial step upon the degradation pathway.

n-Alkanes, with their inert chemical properties and extremely low water solubility, are a group of compounds that possess relatively low biodegradability, especially under anoxic conditions. The sulfate-reducing *Desulfococcus oleovorans* strain Hxd3 is the first strain proved to be able to degrade *n*-alkanes under anoxic conditions. In this thesis, a valid CSIA method for the carbon and hydrogen isotope analysis of *n*-hexadecane was firstly established. The carbon fractionation pattern upon the degradation of *n*-hexadecane by the strain Hxd3 was determined. An insignificant fractionation factor which is typical for the carboxylation mechanism was obtained. However, masking effect might influence the analysis.

A novel method for phenols carbon isotope analysis was successfully established based on liquid chromatography (LC-IRMS). Furthermore, a method for hydrogen isotope analysis was established by combining the acylation of phenols and the application of a Gas Chromatography—Chromium-Based High-Temperature Conversion (Cr/HTC)—Isotope Ratio Mass Spectrometry (GC-Cr/HTC-IRMS) system. Moreover, the first dataset of carbon enrichment factors (ε_c) and apparent kinetic isotope effect values ($AKIE_c$) for different anoxic (and oxic) phenols activation mechanisms was provided.

ZUSAMMENFASSUNG

Kohlenwasserstoffe werden in der Umwelt hauptsächlich biologisch abgebaut. Verglichen mit der Vielzahl von Studien zum aeroben Abbau von Kohlenwasserstoffen, ist weitaus weniger über den anaeroben Abbau bekannt. In der vorliegenden Doktorarbeit wurde der Abbau von BTEX (Benzol, Toluol, Ethylbenzol, Xylole) – insbesondere der des Ethylbenzols, *n*-Alkanen und Phenolen in drei Teilprojekten näher untersucht. Phenolische Verbindungen sind strukturell verwandt zu BTEX-Verbindungen; BTEX und Phenole werden unter aeroben Bedingungen durch identische Mechanismen aktiviert, die beteiligten Enzyme ähneln sich. Die mikrobiellen Abbauwege für *n*-Alkane, TEX und Phenole und die beteiligten Mikroorganismen wurden mittels Analyse stabiler Isotope als hauptsächlichem methodischen Werkzeug näher charakterisiert: Aktivierungsmechanismen wurden anhand des Fraktionierungsmusters von stabilen Kohlenstoff- oder Wasserstoffisotopen bestimmt. Schlüsselorganismen des Abbaus von Ethylbenzol sollten mittels Isotopenmarkierung (stable isotope probing, SIP) identifiziert werden.

Die drei Teilprojekte und die wesentlichen Schlussfolgerungen dieser Doktorarbeit lauten wie folgt:

i) Charakterisierung von ethylbenzolabbauenden, aus kontaminierten Grundwasserleitern hergestellten Anreicherungskulturen unter sulfat- und nitratreduzierenden Bedingungen

ii) Bestimmung des Aktivierungsmechanismus beim Abbau von *n*-Hexadekan durch *Desulfococcus oleovorans* Stamm Hxd3

iii) Entwicklung einer auf komponentenspezifischer Isotopenanalyse basierenden Methode zum Nachweis und zur Quantifizierung von (Alkyl)Phenolen in Umweltproben

Ethylbenzol ist ein in Böden und Grundwässern häufig anzutreffender Grundwasserschadstoff (zumeist im Verbund mit TEX auftretend), verursacht beispielsweise durch Leckagen von Öl- oder Treibstofftanks, Unfällen beim Öl- oder Treibstofftransport oder unsachgemäße Entsorgung von Industrieabfällen. Der mikrobielle Abbau von Ethylbenzol unter strikt anaeroben Bedingungen wurde in Böden und Grundwässern bisher nur selten beobachtet, die verantwortlichen Mikroorganismen

und ihre Abbauwege sind nicht bekannt. In der vorliegenden Arbeit wurde eine anaerobe ethylbenzolabbauende Laborkultur erfolgreich aus Kiessand angereichert; der Sand entstammte einem on-site-Säulensystem, welches mit BTEX-haltigem, sulfidischen Grundwasser an einem Standort in der Nähe von Zeitz, ca. 50 km südwestlich von Leipzig, durchflossen wurde. Der Abbau von Ethylbenzol korrelierte mit der Produktion von Sulfid. Versuche mit ^{13}C-markiertem Ethylbenzol zeigten, dass Ethylbenzol zu CO_2 mineralisiert wurde. Aufgrund des extrem langsamen Wachstums der Kultur konnten die ethylbenzolmetabolisierenden Organismen mittels SIP nicht identifiziert werden. Metagenomische und metaproteomische Studien zeigten, dass Organismen in der Kultur dominierten, die den Phyla Firmicutes, Proteobacteria and Chloroflexi angehörten.

An einem kontaminierten Industriestandort (Òdena) in der Nähe von Barcelona (Spanien) wurde der Abbau von TEX im Grundwasserleiter mittels komponentenspezifischer Isotopenanalyse untersucht. TEX-Proben gewonnen aus Grundwassermesstellen der Quelle und des Abstroms unterschieden sich in der Kohlenstoffisotopensignatur, das Ergebnis weist auf einen mikrobiologischen in situ-Abbau von TEX hin. Aus Grundwasserproben wurden zwei ethylbenzolabbauende nitratreduzierende Organismen angereichert, isoliert und mittels 16S rDNA-Analyse den Gattungen *Azoarcus* und *Thauera* zugeordnet. Die Ergebnisse der komponentenspezifischen Isotopenanalyse weisen darauf hin, dass Ethylbenzol von beiden Kulturen mittels Hydroxylierung der Seitenkette aktiviert wurde.

n-Alkane, insbesondere die längerkettigen, sind verglichen mit BTEX-Verbindungen oder Phenolen sehr schlecht wasserlöslich; die daraus resultierende geringe Bioverfügbarkeit erklärt zum Teil den langsamen Abbau dieser Verbindungen insbesondere unter anaeroben Bedingungen. Der erste Stamm, für den der produktive Abbau von *n*-Alkanen unter anaeroben Bedingungen nachgewiesen werden konnte, war der Sulfatreduzierer *Desulfococcus oleovorans* Stamm Hxd3. In dieser Doktorarbeit wurde eine Methode zur komponentenspezifischen Analyse von Kohlenstoff- und Wasserstoffisotopen von längerkettigen *n*-Alkanen mittels Isotopenverhältnis-Massenspektroskopie und vorgeschalteter gaschromatographischer Auftrennung (Gas chromatograpy-isotope ratio mass spectrometry, GC-IRMS) entwickelt. Anschließend wurde das Kohlenstoff-Fraktionierungsmuster beim Abbau von *n*-Hexadekan durch Stamm Hxd3 bestimmt. Ein

unbedeutender Fraktionierungsfaktor, der für den Carboxylierung typisch ist, wurde erhalten. Der Maskierungseffekt könnte jedoch die Analyse beeinflussen.

Eine neue Methode zur komponentenspezifischen Analyse von Kohlenstoffisotopen in Phenolen mittels vorgeschalteter Flüssigchromatographie (liquid chromatography-isotope ratio mass spectrometry, LC-IRMS) wurde entwickelt. Mit dieser Methode wurden Faktoren für die Anreicherung der Kohlenstoffisotopie (ε_C) und des apparenten kinetischen Isotopeneffekts ($AKIE_C$) für verschiedene aerobe und anaerobe Aktivierungsreaktionen von Phenolen vergleichend bestimmt. Des Weiteren wurde eine neue Methode zur komponentenspezifischen Analyse von Wasserstoffisotopen in Phenolen entwickelt, die auf der Derivatisierung von Phenolen durch Acylierung, nachfolgender gaschromatographischer Auftrennung und anschließender Analyse im Isotopenmassenspektrometer basiert (Gas chromatography-Chromium-Based High-Temperature Conversion (Cr/HTC)-Isotope Ratio Mass Spectrometry, GC-Cr/HTC-IRMS).

1 INTRODUCTION

1.1 Hydrocarbons in the environment: origin, occurrence and extinction

Hydrocarbons are a group of organic compounds which consist of two elements, hydrogen and carbon. Due to the lack of functional groups, most hydrocarbons possess poor water solubility and inert chemical reactivity (Silberberg et al. 2006). The hydrocarbons are usually classified into three categories according to their bonds features: the unsaturated aliphatic hydrocarbons, the saturated aliphatic hydrocarbons and the aromatic hydrocarbons. The aliphatic hydrocarbons can be further divided into straight chain, branched-chain and alicyclic hydrocarbons while the aromatic hydrocarbons have the monocyclic and polycyclic groups (Widdel and Rabus 2001, Silberberg *et al.* 2006). Hydrocarbons are naturally occurring in the environment as metabolites of microorganisms, plants and animals (Widdel and Rabus 2001, Widdel et al. 2006). Furthermore, the the transformation of buried organic material by chemical and microbiological processes into forms like crude oil is another important source of natuarally occurring hydrocarbons (Tissot and Welte 2013). Hydrocarbons played a vital role in the civilization and industrialization process as many human activities rely on the use of hydrocarbon fuels(Widdel and Rabus 2001, Tissot and Welte 2013). However, anthropogenic activity can influence the accumulation and distribution of hydrocarbons in the environment (Tissot and Welte 2013). Well-known examples are oil spills during the manufacture or transportation processes (Swannell et al. 1996).

The inert chemical reactivity of hydrocarbons and their risk to human health has made their environmental fate a great concern. When hydrocarbons enter the environment, a partitioning between water, air and soil occurs. The biodegradation starts in different environment after the partitioning (Knap and Williams 1982). Biodegradation by microorganism represents one of the most important mechanisms by which hydrocarbons are eliminated from water and soil (Council 1985, Aitken et al. 2004). It is therefore vital to understand the degradation pathways and the factors which influence the natural attenuation processes in the environment. On another hand, the study of biodegradation of hydrocarbons contributes to our understanding of the role of microorganisms in the global carbon cycle (Arrigo 2005, Gougoulias et al. 2014, Hutchins and Fu 2017). Furthermore, the decontamination of groundwater and soil using bioremediation has

become a business with rising economic importance (Ammann and Koch 1993, Compernolle et al. 2013, Azubuike et al. 2016) .

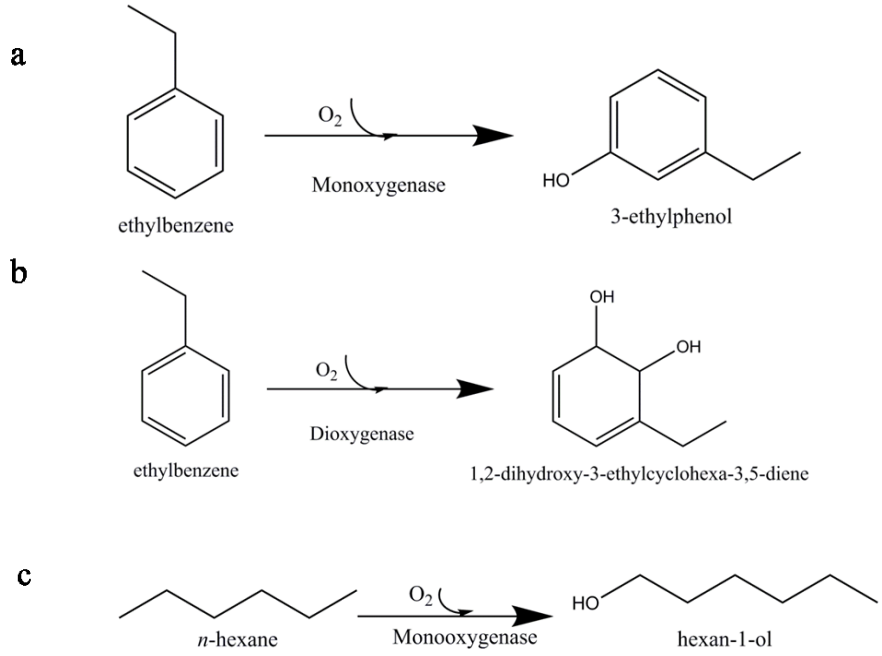
Despite the long recognition and research of this issue, the pathways and factors involved in the biodegradation of hydrocarbons are still not fully understood. Studies rely on concentration measurements and cultivation-based methods which have several limitations. For example, the concentration of one certain contaminant in the field can be affected also by processes such as sorption, water-air partitioning and diffusion (Jeannottat and Hunkeler 2013, Imfeld et al. 2014, Wanner and Hunkeler 2015). Many microbes in the environment are uncultivable or cannot be isolated (Vartoukian et al. 2010, Stewart 2012). Therefore when using the conventional cultivation-based methods in the study of biodegradation, the cultivation and isolation of target microorganisms are challenging (Uhlik et al. 2013, Mishamandani et al. 2014). The stable isotope measurement based methods are in comparison less affected by the mentioned limitation. Ratios of stable isotopes can be used to interpret the degradation pathway of a compound (Elsner et al. 2012, Nijenhuis et al. 2016) and the stable isotope labelling is an excellent way to identify the microorganism which plays the key role of in the given biodegradation (Lueders 2010, Uhlik *et al.* 2013). These methods are introduced in chapter 1.3 of the thesis.

The scope of the current thesis focused on two hydrocarbons, ethylbenzene and *n*-hexadecane as well as the closely relatives of aromatic monohydrocarbons: phenol and cresols. In the following chapter 1.2, the properties of these compounds and their biodegradation are explained in details.

1.2 Biodegradation of ethylbenzene, *n*-alkanes and phenols

There are several common strategies employed by microorganisms in the activation of saturated and aromatic hydrocarbons. In the presence of oxygen, hydroxyl groups are normally introduced using oxygen originating from molecular oxygen and catalyzed by mono- or dioxygenases (Ullrich and Hofrichter 2007, Lewis et al. 2011). The activated oxygen can act as a strong oxidant to overcome the inertness of hydrocarbons. For aromatic compounds, the initial attack can happen either at the aromatic ring or at the side chain (Ullrich and Hofrichter 2007). For *n*-alkanes, the oxidation occurs at the terminal or subterminal position (Rojo 2009, Pérez-Pantoja et al. 2010, Fuchs et al. 2011, Díaz et al. 2013).

Without the presence of oxygen, three mechanisms have been reported: the addition of a fumarate forming the corresponding succinates catalyzed by a group of radical enzymes belonging to the pyruvate formate lyase family (Biegert et al. 1996, Leuthner et al. 1998, Widdel *et al.* 2006, Fuchs *et al.* 2011, Rabus et al. 2016); the introduction of hydroxyl groups using oxygen derived from H_2O catalyzed by the hydroxylase producing the substituted alcohols (Kniemeyer and Heider 2001, Widdel and Rabus 2001, Ulrich et al. 2005, Widdel *et al.* 2006, Fuchs *et al.* 2011); the addition of a carboxyl group catalyzed by carboxylases (Zhang and Young 1997, Meckenstock et al. 2000, Widdel and Rabus 2001, So et al. 2003, Widdel *et al.* 2006, Fuchs *et al.* 2011). A hypothesized pathway of the addition of a methyl group to the hydrocarbon compound upon benzene degradation was proposed by Ulrich et al (Ulrich *et al.* 2005). ^{13}C labeled benzene was used as substrate in the experiment. In return, labeled toluene and benzoate were detected as metabolites. The author concluded therefore a pathway with the methylation of benzene to toluene and the subsequent transformation of toluene to benzoate. Another study using metatransciptome approach, however, did not support this hypothesis (Luo et al. 2014). A carboxylation pathway was proposed for benzene degradation under nitrate-reducing condition instead (Luo *et al.* 2014, Meckenstock et al. 2016). In the figure below (Figure 1.1) are examples showed mechanisms reported for hydrocarbon degradation.



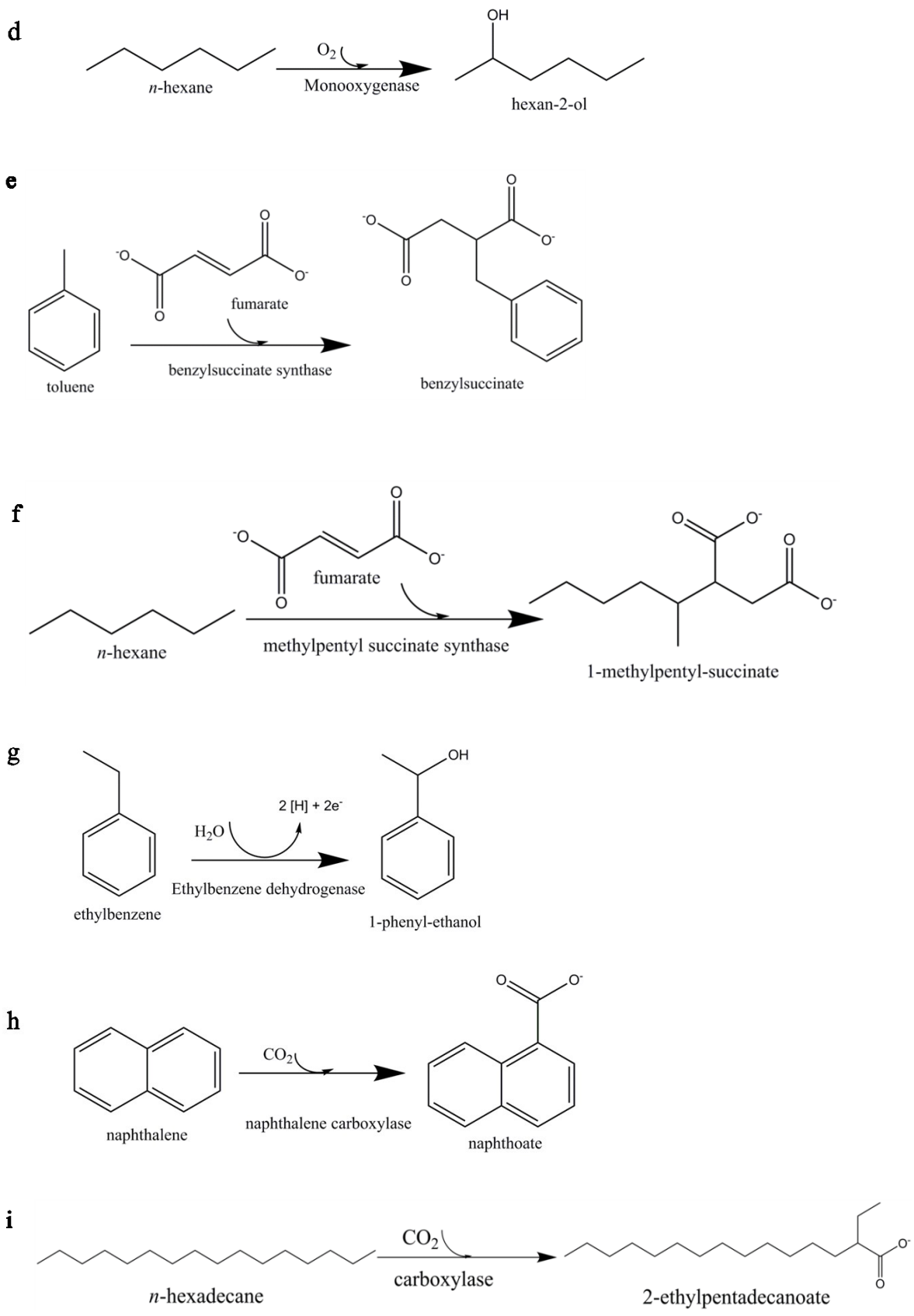


Figure 1.1. The initial reactions upon hydrocarbons degradation. a-d show reactions under aerobic condition and e-i show reactions under anoxic condition. a) Ring hydroxylation of ethylbenzene

(Olsen et al. 1994). b) Ring dihydroxylation of ethylbenzene (Gibson and Parales 2000). c) Terminal monooxygenation of *n*-hexane (Rojo 2009). d) Subterminal monooxygenation of *n*-hexane (Rojo 2009). e) Fumarate addition to toluene (Parales et al. 2008). f) Fumarate addition to *n*-hexane (Rojo 2009). g) Anaerobic hydroxylation of ethylbenzene (Kniemeyer and Heider 2001). h) Carboxylation of naphthalene (Zhang and Young 1997). i) Putative carboxylation of *n*-hexadecane (So *et al.* 2003).

The biochemistry of the biodegradation of the model compounds ethylbenzene, *n*-hexadecane, phenol and cresols are described in detail in the next chapter.

1.2.1 Biodegradation of ethylbenzene

Ethylbenzene, one of the BTEX compounds, occurs naturally as a component of coal tar and petroleum. It is a flammable liquid with a density less than water. It evaporates easily into the air from water and soil and the vapor is heavier than air. It is produced on a large scale as the precursor for the synthesis of styrene, which is used for the manufacture of polystyrene. Ethylbenzene is released to and accumulates in the environment during the manufacture, transportation and waste disposal in the related industrial processes (Welch et al. 2000). Its volatility and relative high water solubility in comparison to other hydrocarbons makes ethylbenzene a common groundwater pollutant (Rabus and Widdel 1995, Wiedemeier et al. 1996, 2002). The risk of ethylbenzene to the environment and human health throw light upon the importance of the evaluation of their degradation process in the environment (ATSDR 2002).

Ethylbenzene has been focused as one of the typical monoaromatic hydrocarbons for the study of biodegradation (Ahlert 1996). However, comparing to the other three compounds of "BTEX", there are relative fewer studies about ethylbenzene degradation especially under anaerobic condition. It has been revealed that ethylbenzene can be mineralized by microorganisms both aerobically and anaerobically. In the presence of oxygen, bacterial monooxygenases and dioxygenases attack the ethyl group or the aromatic ring in the initial step of the degradation process (Jindrova, Chocova et al. 2002). Under anoxic condition, two mechanisms have been shown to initiate the breakdown of ethylbenzene by prokaryotes, namely hydroxylation of the side chain (Figure 1.1 g), and fumarate addition (Foght 2008). The enzyme catalyzing anoxic hydroxylation, ethylbenzene dehydrogenase (EbDH), was firstly detected in the nitrate reducer *Azoarcus* strain EbN1 (Rabus and Heider 1998) and has been studied in detail (Kniemeyer and Heider 2001,

Szaleniec, Borowski et al. 2010, Knack, Hagel et al. 2012). The EbDH is a molybdenum, iron-sulfur cluster and heme containing enzyme which catalyze the oxidation of ethylbenzene at the benzylic carbon atom leading to the product of (S)-1-phenylethanol (Kniemeyer and Heider 2001). Fumarate addition as initial activation reaction was detected in a marine sulfate reducer (Kniemeyer, Fischer et al. 2003), but the responsible enzyme has not been characterized yet. *Georgfuchsia toluolica* strain G5G6 is reported to be able to couple ethylbenzene mineralization to ferric iron reduction (Weelink et al. 2009, Dorer et al. 2016). Two other ethylbenzene-degrading enrichment cultures under iron-reducing conditions were described and it is hypothesized that dissimilatory iron reduction was coupled to the ethylbenzene degradation (Jahn et al. 2005, Weelink et al. 2010). The reported anaerobic ethylbenzene-degrading cultures/strains are listed in Table 1.1.

Table 1.1 Anaerobic ethylbenzene-degrading cultures/strains

Culture/Strain	Electron acceptor	Activation step	References
Aromatoleum aromaticum EbN1	nitrate, manganese	anaerobic hydroxylation	(Rabus and Widdel 1995, Kniemeyer and Heider 2001)
enrichment culture	nitrate	unknown	(Hutchins 1991, Hutchins et al. 1991)
Strain RCB	nitrate	unknown	(Chakraborty et al. 2005)
Strain JJ	nitrate	unknown	(Coates et al. 2001)
Strain EB1	nitrate	anaerobic hydroxylation	(Ball et al. 1996)
Georgfuchsia toluolica G5G6	nitrate, manganese, ferric iron	anaerobic hydroxylation	(Dorer et al. 2014)
DD-Anox 1, enrichment culture dominated by *Azoarcus sp.*	nitrate, manganese	anaerobic hydroxylation	(Dorer *et al.* 2014)
ebenz K21, enrichment culture	ferric iron	unknown	(Jahn *et al.* 2005)
Strain EbS7	sulfate	fumarate addtion	(Kniemeyer et al. 2003)

Zz3, enrichment culture	sulfate	putative fumarate addition	(Dorer *et al.* 2014)
enrichment culture	sulfate	unknown	(Gieg et al. 1999)
enrichment culture	methanogenic	unknown	(Wilson et al. 1986)
DD-Anox 2, enrichment culture	unknown electron acceptor	putative fumarate addition	(Dorer *et al.* 2014)

Although ethylbenzene and toluene are chemically very similar to each other, there were much less ethylbenzene degraders reported than toluene degraders. The pathway of anaerobic toluene degradation has been intensively studied and only one activation mechanism was confirmed so far: the addition of toluene to fumarate resulting in the formation of benzylsuccinate (Leuthner *et al.* 1998). Hydroxylation was not found as the initial step upon toluene degradation in comparison. The enzyme EbDH, which plays the key role in the ethylbenzene hydroxylation pathway, was found to be inhibited by the addition of toluene (Heider et al. 2016).

1.2.2 Biodegradation of *n*-alkanes

Aliphatic saturated hydrocarbons (alkanes) are the most abundant hydrocarbons in crude oil. They also belong to the most inactive chemical compounds and their inertness increases with the chain-length. In most cases, an activation factor like high temperature or catalysts is needed for the participation of alkanes in chemical reactions (Rehm and Reiff 1981, Fessenden and Fessenden 1982). Despite their low water solubility, low chemical reactivity and the trendiness to accumulate in cell membranes, biodegradation of alkanes plays an important role in their transformation in natural environment (Callaghan et al. 2006, Callaghan et al. 2009). *n*-Hexadecane is chosen in this study as it is a typical representative of the alkanes with medium chain length and low water solubility as well as low chemical reactivity.

The aerobic biodegradation of *n*-alkanes has been well described. In the presence of molecular oxygen (O_2), terminal, subterminal or biterminal hydroxylations are catalyzed by mono- or dioxygenases (Figure 1.1) (Maeng et al. 1996, Gibson and Parales 2000). Hydroxyl groups are formed using oxygen derived from O_2. The yielding substituted alcohol, in the case of terminal hydroxylation, goes through further oxidation to fatty acids and is then degraded through the beta-oxidation (Rehm and Reiff 1981, Watkinson

and Morgan 1990). Anaerobic *n*-alkane degradation has been reported to be coupled with sulfate or nitrate reduction (Aeckersberg et al. 1991, So and Young 1999, Ehrenreich et al. 2000, So et al. 2003, Kniemeyer et al. 2007). In addition, *n*-alkanes degradation has also been reported under methanogenic conditions (Zengler et al. 1999, Alain et al. 2006). Two activation mechanisms have been described: the subterminal addition of a fumarate yielding the substituted succinate and the hypothesized activation mechanism by subterminal carboxylation (So and Young 1999b, So et al. 2003). The products of the activation steps undergo further bonds rearrangement or oxidation before they are metabolized through the beta-oxidation pathway (So et al. 2003, Callaghan et al. 2006, Callaghan et al. 2009). Besides, an intra-aerobic degradation pathway was proposed for methane as well as medium to long-chain *n*-alkanes degraded under nitrate-reducing conditions. The molecular oxygen which is produced through the disproportionation of nitrous oxide (produced by teduction of nitrate to nitrite) is used subsequently for hydrocarbon activation (Ettwig et al. 2010, Zedelius et al. 2011). The sulfate-reducer *Desulfococcus oleovorans* strain Hxd3 is the first described isolated strain which is capable of anaerobic *n*-alkanes degradation (Aeckersberg et al. 1991). A hypothesized carboxylation at the C3 position of *n*-hexadecane has been proposed by So and colleagues by using ^{13}C-labelled studies of lipids (So et al. 2003). An alternative hydroxylation activation mechanism has been proposed for the n-hexadecane degradation by strain Hxd3 as genome analysis reveals no genes for a fumarate-addition enzyme but genes coding for a EbDH-like enzyme (Heider and Schühle 2013). Biochemical studies indicated that anaerobic degradation of *n*-alkane is initiated by hydroxylation at the subterminal carbon atom (Sünwoldt, unpublished results, personal communication, (Heider et al. 2016)). The reported anaerobic n-hexadecane-degrading cultures/strains are listed in Table 1.2.

Table 1.2 Anaerobic *n*-hexadecane-degrading cultures/strains

Culture/Strain	Electron acceptor	Activation step	References
enrichment culture	nitrate	putative carboxylation	(Callaghan *et al.* 2009)
Strain HdN1	nitrate	unknown	(Ehrenreich *et al.* 2000)
Strain Hxd3	sulfate	putative carboxylation/ hydroxylation	(Aeckersberg *et al.* 1991, Callaghan

			2013, Heider *et al.* 2016)
Desulfatibacillum aliphaticivorans Strain CV2803	sulfate	putative fumarate addtion	(Cravo-Laureau et al. 2005)
Archaeoglobus fulgidus strain VC-16	sulfate/thiosulfate	putative fumarate addition	(Khelifi et al. 2014)
Strain TD3	sulfate	unknown	(Rueter et al. 1994, Wilkes et al. 2016)
Strain AK-01	sulfate/sulfite /thiosulfate	fumarate addition	(So and Young 1999, Callaghan et al. 2008)
enrichment culture	methanogenic	unknown	(Jones et al. 2008)
enrichment culture	methanogenic	unknown	(Anderson and Lovley 2000)
enrichment culture	methanogenic	unknown	(Zengler et al. 1999)
enrichment culture	methanogenic	unknown	(Siddique et al. 2011)

1.2.3 Biodegradation of phenol and cresols

Phenol and cresols are simple-structured phenols, having both natural and anthropogenic origins. These compounds are usually obtained from petroleum or coal tar and are widely used in chemical, petrochemical as well as pharmaceutical industries. Their presence as pollutants in air and water is therefore also related with these industrial processes (Marrot et al. 2006). The increasing presence of phenols in the environment and their toxicity creates health concerns as phenol and cresols have a corrosive effect when in contact with skin and mucosal membranes. In addition, para-cresol (*p*-cresol) is classified as probable carcinogenic for humans (Kahru et al. 2002, Michałowicz and Duda 2007). Phenol and cresols are highly water soluble (phenol, 83g/l; cresol isomers, 22-26g/l) (Yalkowsky et al. 1987) leading to higher environmental risks as these compounds can be transported in aqueous systems (e.g., groundwater) over long distances in high concentrations.

Biodegradation of phenol and cresols occurs under both aerobic and anoxic conditions. Under aerobic conditions, phenol hydroxylase initiates the degradation by the

hydroxylation of phenol to catechol using molecular oxygen as a co-substrate. This is followed by the aromatic-ring breakdown by either *ortho* or *meta* cleavage (Powlowski and Shingler 1994, Ehrt et al. 1995, Mahiudddin et al. 2012). The aerobic degradation of cresols proceeds via either ring- or methyl-hydroxylation catalyzed by phenol hydroxylases (Hopper and Taylor 1975, Powlowski and Shingler 1994, Paller et al. 1995, Solyanikova and Golovleva 1999). During anaerobic degradation of phenol, phenol is firstly carboxylated to 4-hydroxybenzoate prior to the ring cleavage (Tschech and Fuchs 1987, Gallert and Winter 1992, Li et al. 1996, Shinoda et al. 2000). It was demonstrated that the first step of the carboxylation procedure is an ATP-dependent phosphorylation of the hydroxyl group catalyzed by phenylphosphate synthase (Lack and Fuchs, 1994; Schmeling et al., 2004); subsequently, the formed phenylphosphate is carboxylated to 4-hydroxybenzoate under release of phosphate by phenylphosphate carboxylase (Lack et al., 1991; Lack and Fuchs, 1992; Schühle et al., 2004). The initial reactions upon cresols degradation under anoxic condition include O_2-independent hydroxylation, carboxylation and fumarate addition. During O_2-independent hydroxylation, the methyl group carbon is hydroxylated by hydroxyl ions stemming from H_2O, leading to the intermediate hydroxybenzyl alcohol (McIntire et al. 1985, Hopper et al. 1991). The fumarate addition reaction upon cresols degradation was most well studied in the sulfate reducer *Desulfosarcina cetonica*; during *p*-cresol and *m*-cresol degradation, typical metabolites of the fumarate addition pathway, 3-hydroxybenzylsuccinate and 4-hydroxybenzylsuccinate were detected, respectively. The reaction was proposed to be initiated by abstraction of an H atom from the methyl group by an enzyme-bound glycine radical. The formed hydroxyl benzyl radical attacks the fumarate double bond and an H atom was obtained for the formation of hydroxylbenzylsuccinate synthase (Müller et al. 1999, Müller et al. 2001). An overview of the activation step upon microbial phenol and cresol degradation is shown in chapter 3.1.1 in the results part.

1.3 Stable isotope based methods for the assessment of hydrocarbon degradation

1.3.1 Compound specific stable isotope analysis (CSIA) of hydrocarbons

CSIA is a method that analyzes the naturally occurring stable isotopes and it is an effective tool in the monitoring of environmental contaminants. The target compounds are chromatographically separated by gas chromatography (GC) or liquid chromatography (LC) prior to the isotopic analysis by isotope ratio mass spectrometry (IRMS) (Hunkeler

and Bernasconi, 2009). The method was developed in the late 1970s by the working group of Hayes and collaborators (Matthews and Hayes 1978, Merritt et al. 1994). The coupling of GC to IRMS started already from the late 1970s (Matthews and Hayes 1978) while the coupling of LC to IRMS was firstly described in 2005 (Mohammadzadeh et al. 2005). The application of CSIA in the study of bioremediation started from the late 1990s. Meckenstock and collegues studied the carbon fractionation upon microbial degradation of toluene by four strains and obtained similar enrichment factors (-1.5‰, -1.7‰, -1.7‰ and -1.8‰) for all the strains (Meckenstock et al. 1999). Dayan and collegues evaluated the carbon isotopic fractionaltion during reductive dehalogenation of chlorinated ethenes under iron-reducing conditions and observed large $\delta^{13}C$ values shifts (12‰ - 24‰) upon 80-90% degradation (Dayan et al. 1999). Sherwood Lollar and collegues investigated carbon isotope fractionation during the biodegradation of trichloroethylene (TCE) as well as toluene. They found out an enrichment of over 14‰ upon anaerobic degradation of TCE while no obvious fractionation upon aerobic toluene degradation (Sherwood Lollar et al. 1999). Stehmeier and colleagues applied CSIA to monitor the carbon isotope fractionation during laboratory degradation of benzene and styrene by microcosms as well as in-situ remediation of several hydrocarbons. They concluded that there were enrichment in ^{13}C of the residual hydrocarbons in both laboratory and in-situ degradation processes (Stehmeier et al. 1999). CSIA are utilized in the monitoring of biodegradation of various environmental pollutants ever since. It can be used for (i) identifying pollution sources by detecting small variations in the isotopic composition of compounds, and for (ii) assessing the biodegradation of pollutants in the environment by detecting the associated isotope fractionation (Aravena and Hunkeler, 2009; Elsner et al., 2005). It has been proven to be successful in the monitoring of biodegradation of rather non-polar compounds such as hydrocarbons (e.g., BTEX) (Vogt et al. 2016) and chlorinated organics (Nijenhuis *et al.* 2016) as well as several pesticides and pharmaceuticals (Reinnicke et al. 2012, Elsner and Imfeld 2016, Schurner et al. 2016). In the current thesis, CSIA was applied in analyzing the biodegradation of selected hydrocarbons and phenols.

CSIA with regard to biodegradation takes advantage of different reaction rates of isotopologues in rate-limiting steps of (bio)chemical reactions. Due to energetic constraints, the lighter isotopologue reacts usually slightly faster resulting in a residual substrate pool enriched in heavy isotopes at the reactive position in the course of the

reaction; correspondingly, the product becomes isotopically lighter. This is termed kinetic isotope effect (KIE). KIEs can be calculated by forming the ratio of the reaction rates of the heavy and light isotopologues, or by applying the Rayleigh equation which relates changing isotope signatures and concentrations of the substrate. By the latter procedure, apparent kinetic isotope effects (AKIEs) are usually derived in studies dealing with the (bio)transformation of environmental relevant pollutants (Elsner et al., 2005). KIEs can be further divided into primary and secondary isotope effects. Isotope effects related to the breakage or formation of chemical bonds to the atom of interest in the reacting substrate are termed primary isotope effects. Isotope effects occurring in the absence of bond breakage or formation in the rate-determining step of a reaction are termed secondary isotope effects. Secondary isotope effects are usually by at least one magnitude lower than primary effects (Elsner et al., 2005). The larger the mass difference between two isotopes is, the larger the KIE is (Elsner et al. 2005). Due to the significant mass difference between ^{1}H and ^{2}H, the secondary isotope effect of hydrogen isotopes can result in a considerable KIE value. Different types of reactions may lead to isotope fractionation with different extent due to the energy level differences during the bonds dissociation and formation processes in the transition state. Thus, analysis of stable isotope fractionation during biodegradation of a certain compound may indicate the biochemical mechanism by which the compound is initially transformed, hence indicating the biodegradation pathway (Vogt *et al.* 2016). Multi-element compound-specific isotope fractionation (ME-CSIA) has been a promising tool in identifying the initial activation of hydrocarbons. The concept was first introduced for the determination of MTBE biodegradation pathways in groundwater (Kuder et al. 2005, Zwank et al. 2005). The analysis of the isotope of two or more elements can effectively avoid the masking effects of single element isotope analysis (Vogt *et al.* 2016).

For quantifying in situ biodegradation by CSIA and for identifying distinct reaction mechanisms, it is essential to determine the magnitude of isotope fractionation of the first step of known pollutant degradation pathways in laboratory experiments using model cultures. In recent years, isotope fractionation factors for the biodegradation of various pollutants and degradation pathways have been published, using stable isotopes of several elements (e.g., D/H, $^{13}C/^{12}C$,) depending on the compounds structure (Musat et al. 2016, Nijenhuis *et al.* 2016, Schurner *et al.* 2016). Below is an overview of previous studies in

which carbon and hydrogen enrichment factors and apparent kinetic isotope effects values for different hydrocarbon and phenols activation reactions are given.

Table 1.3 Ranges of isotope fractionation upon hydrocarbons and phenols biodegradation

Benzene					
Reaction	$\varepsilon_{bulk,c}$ *	$AKIE_C$*	$\varepsilon_{bulk,H}$*	$AKIE_H$*	Lambda (Λ)*
Dihydroxylation	-1.3 to -0.7	1.002 to 1.004	n.d.	0.985 to 1.015	±5 to ±7
Ring hydroxylation	-4.3 to -1.5	1.005 to 1.016	-18 to -11	1.036 to 1.121	3 to11
Carboxylation	-3 to -2.8	1.017 to 1.018	-56 to -47	1.39 to 1.51	16 to 17
Toluene					
Reaction	$\varepsilon_{bulk,c}$	$AKIE_C$	$\varepsilon_{bulk,H}$	$AKIE_H$	Lambda (Λ)
Dihydroxylation	-1.8 to -0.4	1.001 to 1.006	-28 to -2	1.011 to 1.029	-1 ± 2
Ring hydroxylation	-1.1	1.008	-16	1.016	n.d.
Side-chain hydroxylation (reaction with molecular oxygen)	-3.3 to -0.4	1.0004 to 1.024	-956 to 159	1.026 to 27.7	16 to 68
Fumarate addition	-6.7 to 3.8	1.004 to 1.046	-126 to -135	1.2 to 4.7	-26 to 41
Ethylbenzene					
Reaction	$\varepsilon_{bulk,c}$	$AKIE_C$	$\varepsilon_{bulk,H}$	$AKIE_H$	Lambda (Λ)
Dihydroxylation	-0.5 ± 0.1	1.002	4 ± 3	1	-7 ± 3
Ring hydroxylation	-0.6 to -0.4	1.003 to 1.005	-6 to -2	1.0 to 1.1	5 to 9
Side-chain hydroxylation (reaction with H_2O)	-4.1 to -1.3	1.01 to 1.033	-111 to -50	1.5 to 4.4	18 to 23
Fumarate addition	-0.7 to -0.6	1.005 to 1.006	-96 to -76	1.8	168 to 278
Xylene					
Reaction	$\varepsilon_{bulk,c}$	$AKIE_C$	$\varepsilon_{bulk,H}$	$AKIE_H$	Lambda

					(Λ)
Side-chain hydroxylation (reaction with molecular oxygen)	-2.3 to -1.7	1.014 to 1.019	n.d.	n.d.	n.d.
Fumarate addition	-2.7 to -0.7	1.006 to 1.022	-50 to -19	1.23 to 1.93	12 to 29
Methane					
Reaction	$\varepsilon_{bulk,c}$	$AKIE_C$	$\varepsilon_{bulk,H}$	$AKIE_H$	Lambda (Λ)
Side-chain hydroxylation (reaction with molecular oxygen)	-29.2 to -14.8	1.015 to 1.029	-232 to -110	1.7 to 13.5	7.3 to 10.5
Activation by coenzyme M	-35.7 to -11.9	1.012 to 1.037	-229.6 to -100.7	1.7 to 12.2	7.7 to 9.3
Propane					
Reaction	$\varepsilon_{bulk,c}$	$AKIE_C$	$\varepsilon_{bulk,H}$	$AKIE_H$	Lambda (Λ)
Fumarate addition	-8.7 to -2.6	1.008 to 1.026	-92 to -16	1.14 to 2.14	6.3 to 11.9
n-Butane					
Reaction	$\varepsilon_{bulk,c}$	$AKIE_C$	$\varepsilon_{bulk,H}$	$AKIE_H$	Lambda (Λ)
Fumarate addition	-5.6 to -0.8	1.003 to 1.023	-47 to -5	1.05 to 1.78	4.9 to 8.7
Phenols					
Reaction	$\varepsilon_{bulk,c}$	$AKIE_C$	$\varepsilon_{bulk,H}$	$AKIE_H$	Lambda (Λ)
Side-chain hydroxylation (reaction with molecular oxygen)	-1.5 to -1.4	1.009 to 1.01	n.d.	n.d.	n.d.
Side-chain hydroxylation (reaction with	-3.6 to -2.3	1.016 to 1.026	n.d.	n.d.	n.d.

H_2O)					
Fumarate addition	-3.9 to -1.6	1.011 to 1.028	n.d.	n.d.	n.d.
Carboxylation	-0.7 to 0.4	0.998 to 1.004	n.d.	n.d.	n.d.

* $\varepsilon_{bulk,c}$, $\varepsilon_{bulk,H}$: bulk carbon or hydrogen enrichment factor (calculation is shown in chapter 2.3.3.7);

AKIEc, $AKIE_H$: apparent carbon or hydrogen kinetic isotope effect (calculation is shown in chapter 2.3.3.7);

Lambda (λ) $\approx \varepsilon_{bulk,c} / \varepsilon_{bulk,H}$

(Fischer et al. 2008, Vogt et al. 2008, Morasch et al. 2002, Dorer et al. 2014, Kümmel et al. 2016, Pati et al. 2016, Dorer et al. 2014, Wei et al. 2016, Mancini et al. 2006, Feisthauer et al. 2011, Rasigraf et al. 2012, Meckenstock et al. 1999, Kümmel et al .2013, Tobler et al. 2007, Tobler et al. 2008, Morasch et al . 2001, Herrmann et al. 2009, Ahad et al. 2000, Richnow et al. 2003, Griebler et al. 2004, Jaekel et al. 2014, Kniemeyer et al. 2007, Vieth and Wilkes 2006, Bergmann et al. 2011, Holler et al. 2009)

While numerous studies reported isotope fractionation for BTEX compounds, in contrast, there is only a few studies about the isotope fractionation of alkanes to date (Meckenstock et al. 2004, Elsner *et al.* 2005, Bouchard et al. 2008). Bouchard and colleagues (2008) reported carbon isotope fractionation data upon the degradation of propane to *n*-decane aerobically by soil microcosms. They observed decreasing of enrichment factors from propane to *n*-decane and no measurable enrichment was expected for *n*-alkanes with chain lengths longer than C_{10}. The main reason for this phenomenon is that the dilution effect becomes more obvious with the increase of chain length. Moreover, transport as well as enzyme binding steps become more rate limiting (Bouchard *et al.* 2008). The stable isotope fractionation associated with anaerobic degradation of *n*-alkanes has been well studied only for propane and *n*-butane (Kniemeyer *et al.* 2007, Jaekel et al. 2014). Pure and enriched cultures which degrade medium to long chain *n*-alkanes are very limited (Callaghan 2013, Musat *et al.* 2016). Except for the challenges of isotope fractionation determination mentioned for aerobic *n*-alkane degradation, the difficulty in cultivation and development of analytical methods with high sensitivity remain two other challenges for the characterization of isotope fractionation upon anaerobic degradation of *n*-alkanes with longer carbon chains (Musat *et al.* 2016).

1.3.1.1 Analysis of stable carbon and hydrogen isotopes by gas chromatography—isotope ratio mass spectrometry (GC-IRMS)

The isotope signatures of hydrocarbons that can be introduced into a GC-column are usually analyzed by methods based on gas chromatography–isotope ratio mass spectrometry (GC-IRMS) (Hunkeler and Bernasconi, 2009). The samples are separated by a GC. After this, using IRMS, the molecules are firstly introduced into the ion source. The ionized molecules go across a magnetic field where they are deflected with different radiuses depending on their mass/charge ratio. Afterwards, the molecules are collected in Faraday cups and the abundance of the isotopes is measured in the form of molecules which contain the element of interest. The working mechanism of a GC-IRMS is illustrated in Figure 1.2. The low abundance of some natural heavy isotopes (i.g $^{2}H:^{1}H=99.99:0.01$) is a limiting factor of CSIA (Hunkeler and Elsner 2009). In the analysis of environmental samples containing low concentrated hydrocarbons, a major challenge is, especially in the analysis of hydrogen isotope, to introduce a sufficient amount of analytes, into the IRMS (Vogt *et al.* 2016). The amount of samples to provide a precise measurement of the isotope ratios is defined as the Method Detection Limit (MDL). The MDL of carbon isotopes for BTEX compounds is around one µg/L using the standard purge and trap system (Zwank et al. 2003). For hydrogen isotopes, the MDLs are about 30 times higher than carbon isotopes (Hunkler and Bernasconi 2010). To lower the MDL, several methods for sample extraction prior to the injection into GC injector have been described such as headspace direct injection, solid phase microextration (SPME), in-needle extraction (Zwank *et al.* 2003). In the scope of this thesis, the methods of liquid-liquid extraction, SPME as well as headspace-programmed temperature vaporizer (HS-PTV) have been employed.

Solvent extraction is the most commonly used method for the preconcentration of samples prior to the isotope analysis (Hunkeler and Bernasconi 2009). For instance, pentane extraction is widely used in the sample preparation for BTEX compounds. The corresponding MDLs has been reported as 0.1mg/L (Dempster et al. 1997).

Solid-phase microextraction (SPME) has been developed as a fast and solvent-free injection method which is suitable for compounds with a relatively high octanol-water partition coefficient (Zhang et al. 1994, Dias and Freeman 1997, Zwank *et al.* 2003). In this method, the analytes are absorbed by the sorbent in the SPME fiber and then directly desorbed in the GC injector. According to the phase of which the SPME fibers take samples, the method can be distinguished as liquid phase sampling SPME and headspace sampling SPME. In the liquid phase sampling SPME method, SPME fibers are inserted

into liquid samples containing NaCl for the extraction (Achten and Püttmann 2000, Dewsbury et al. 2003). This method however, leads always to a shorter life-time of the fibers due to exposure to salt (Cassada et al. 2000).

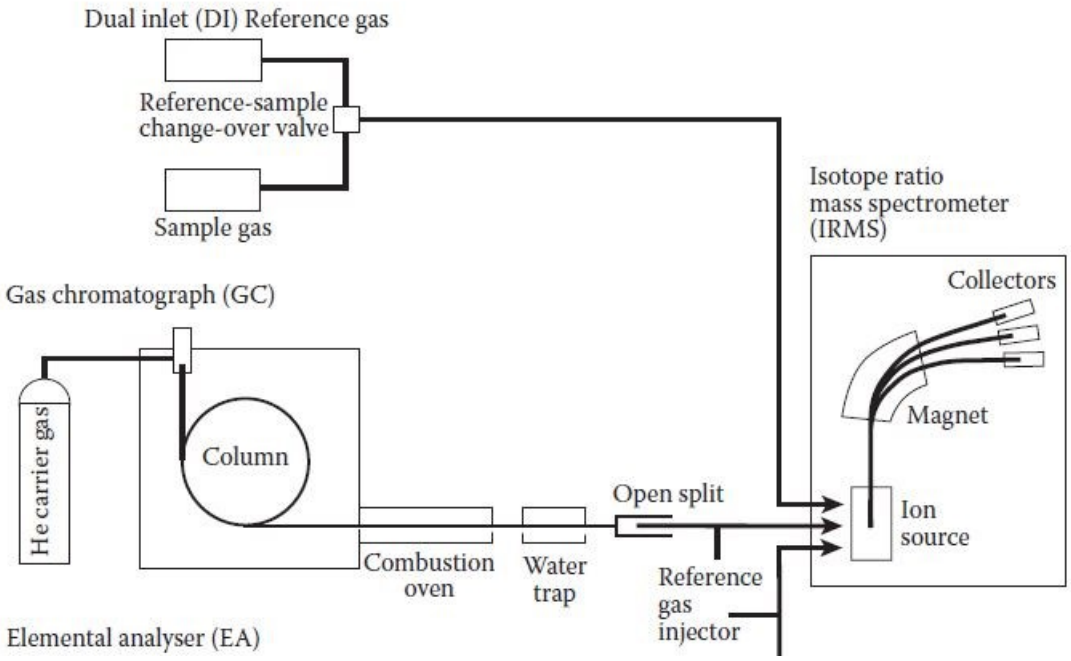


Figure 1.2 The working mechanism of GC-IRMS, cited from chapter 2 of the book "Environmental isotopes in biodegradation and bioremediation" (Aelion et al. 2010) .

In the alternative headspace sampling SPME (HS-SPME) method in comparison, the fiber is immersed into the headspace of a partially filled headspace vial for the extraction (Hunkler et al. 2001, Gray et al. 2002).The SPME method has been used for the analysis of many organic compounds. However, the sample treatment process of this method can lead to slight isotope fractionation. The MDL of the SPME method for carbon stable isotope analysis has been reported as 4-2200 µg/L analytes in water samples (Dias and Freeman 1997, Hunkler *et al.* 2001, Gray *et al.* 2002, Palau et al. 2007, Rakoczy et al. 2011). The HS-SPME method is used in this thesis for the analysis of BTEX hydrogen stable isotope signatures in groundwater samples.

The HS-PTV described by Herrero-Martín and colleagues is an alternative preconcentration method developed for typical groundwater contaminants such as MTBE and BTEX at microgram per liter concentrations (Herrero-Martin et al. 2015). In this method, a relatively larger volume (maximum 5 mL) of the headspace samples from

water samples is injected into the pre-cooled injector (0 °C, pre-cooled by liquid nitrogen). The volatile analytes are then being trapped at 0 °C into a sorbent attached to the GC liner (Pérez Pavón et al. 2007, Pérez Pavón et al. 2008, Herrero-Martin *et al.* 2015). The water vapor produced in the process is controlled at the same time with a split line. The GC-liner is then fast heated to 300 °C for desorption of the analytes into the GC column. The method provides a possibility of accurate analysis of water samples in lower micrograms per liter. The MDLs has been reported as 2-60 µg/L for carbon stable isotope analysis and 60-97 µg/L for hydrogen in the investigated compounds (MTBE and BTEX) (Herrero-Martin *et al.* 2015). The HS-PTV method has been tested in this thesis for the analysis of hydrogen stable isotopes of BTEX compounds in groundwater samples in lower micrograms per liter.

The analysis methods of stable isotopes of *n*-alkanes vary, as their physical and thermochemical properties change with the increase of chain length. Propane and butane are gaseous at standard conditions (0 °C, 100 kPa). The biodegradation takes place with the part of compounds which dissolves in water. Jaekel and collegues studies extensively the carbon and hydrogen isotope fractionation upon the biodegradation of propane and butane. In their studies, the gaseous *n*-alkanes were added as substrate and gas-phase samples from the culture were taken and measured by headspace-GC and GC-IRMS (Savage et al. 2010, Jaekel et al. 2013, Jaekel *et al.* 2014). Bouchard and collegues reported the carbon stable isotope fractionation upon the aerobic degradation of *n*-alkanes with chain length from C3 to C10. Cultavition were performed in containers with large head space and the substrate *n*-alkanes were added directly to the culture. The head space were sampled for subsequent measurement (Bouchard *et al.* 2008). In the case of longer-chain *n*-alkanes (from C11), the substrates are extracted by solvents from the liquid cultures prior to the analysis (Duan and He 2011).

1.3.1.2 Compound-specific carbon isotope analysis of phenol and cresols via liquid chromatography—isotope ratio mass spectrometry (LC-IRMS)

Sensitive analysis of polar compounds like phenols by GC-IRMS is virtually impossible due to their physical-chemical characteristics: they are hydrophilic and thermo-labile compounds. Therefore, phenols are usually chemically derivatized by e.g. silylation or esterification prior to analysis by GC (Pyon et al., 1997; Zhang et al., 2005). However, with regard to analysis of stable isotopes, any derivatization procedure increases the

uncertainty of carbon isotope analysis as additional carbon atoms are introduced (Rieley, 1994). An alternative method for isotope analysis of polar compounds has become available after commercialization of liquid chromatography–isotope ratio mass spectrometry (LC-IRMS). In a LC-IRMS system, the analytes which still dissolved in the aqueous phase are separated by liquid chromatography and subsequently quantitatively converted into CO_2 via wet oxidation with sodium persulfate in the presence of phosphoric acid (Krummen et al., 2004).

1.3.1.3 Compound-specific hydrogen isotope analysis of phenols via acylation and gas chromatography—chromium-based-high-temperature conversion—isotope ratio mass spectrometry (GC-Cr/HTC-IRMS)

The LC-IRMS system described in chapter 1.3.1.2 offers a good solution for the carbon isotope analysis of phenols. However, the hydrogen isotopes of phenols cannot be analyzed by this method due to the limitation in the choice of eluent for the LC system. The conventional GC-IRMS method is not applicable for the hydrogen isotope analysis of phenols because of large variations of isotope signatures in phenol standards with different concentrations (Dorer & Vogt, personal communication). Therefore, a conventional derivatization step to modify the chemical properties of phenols prior to the hydrogen isotope analysis is proposed in this thesis. The acylation reagent trifluoroacetic anhydride (TFAA) is selected as the derivatization reagent for the following reasons: 1) the derivatization procedure is relatively easy and fast; 2) the fluorinated reagent is able to introduce electron-capturing properties and increase the volatility of the original compounds (Kataoka 1996). The chemistry of the process is as described below:

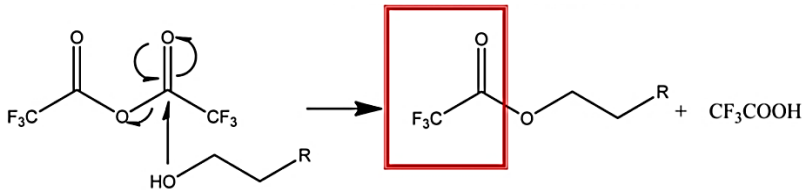


Figure 3.3. Acylation reaction with–OH end groups and TFAA for the formation of trifluoroacetate end groups (Kagawa 2013).

With the conventional GC-IRMS system with high-temperature conversion (HTC), in principle, organic compounds which can be introduced into a GC column and meet the MDL requirements can be analyzed. However, with the presence of other elements such as Cl, F and S, the conversion process can be interfered (Hunkeler and Bernasconi 2009).

In the hydrogen isotope analysis, possible fractionation bias will also be generated due to the formation of byproducts such as HCl (Kelly et al. 2001, Hitzfeld et al. 2011, Hunsinger et al. 2013). To overcome this bottleneck in the analysis of compounds containing other atoms (N, S, F, Cl etc), methods were developed based on the incorporation of a chromium reactor. The hydrogen-bearing byproducts react with the hot chromium and the hydrogen is in this way released again as H_2 (e.g $2Cr + 6HCl \rightarrow 2CrCl_3 + 3H_2$). In this way, the hydrogen isotopic composition of the analytes will not be affected by the byproducts (Kelly *et al.* 2001, Morrison et al. 2001). On the basis of this concept, a Chromium-Elemental Analyzer has been developed by Gehre and colleagues for the accurate analysis of hydrogen isotopes for compounds containing heteroelements such as Cl or F (Gehre et al. 2015). Based on this system, a Gas Chromatography—Chromium-Based High-Temperature Conversion (Cr/HTC)—Isotope Ratio Mass Spectrometry (GC-Cr/HTC-IRMS) was introduced by Renpenning and colleagues to enable the compound-specific hydrogen isotope analysis of the mentioned analytes (Renpenning et al. 2015). The system is used in this thesis for the analysis of hydrogen stable isotope of fluorinated derivatives of phenols.

1.3.2 Stable isotope probing (SIP)

The identification of physiologically distinct functions in microorganisms by classical microbiological methods is dependent on the isolation and establishment of a pure culture (Koch 1881). However, the majority of microorganisms is not cultivable by the use of classical cultivation approaches, thus the application spectrum of the conventional method in elucidating the functionality of a certain spices is limited (Pace 1997, Keller and Zengler 2004) . In recent years, a cultivation independent method to link taxonomic identity and functional properties of microorganism communities has been developed. The method is based on the incorporation of isotopically labelled (e.g ^{13}C, ^{15}N) substances into the biomass during growth. The incorporation process and the name of the method are thereafter defined as “stable isotope probing” (SIP) (Boschker et al. 1998, Radajewski et al. 2000, Manefield et al. 2004). According to the different biomolecules used for the detection, the method can be further categorized into four divisions: the nucleic acid-based SIP, lipids- based SIP, protein-based SIP and the single-cell-SIP using nanoscale secondary ion mass spectrometry (nanoSIMS) or confocal Raman microscopy (Boschker et al. 1998, Radajewski *et al.* 2000, Manefield et al. 2002, Neufeld et al. 2007,

Jehmlich et al. 2008, Jehmlich et al. 2010, Kuppardt et al. 2010, Seifert et al. 2012, Vogt *et al.* 2016).

Protein-SIP is a relatively new method in the identification of isotopically enriched proteins in microorganisms after incubation with a ^{13}C or ^{15}N labeled substrate. The isotopically enriched proteins can be further used for the identification of certain metabolic activities and the acquirement of taxonomic information (Cargile et al. 2004, Snijders et al. 2005, Jehmlich *et al.* 2008, Seifert *et al.* 2012, Taubert et al. 2012, Starke et al. 2016). In comparison with nucleic acid based SIP, the problem of low resolution between labelled and non-labelled nucleic acids and requirement of relatively high incorporation levels is avoided. For the analysis of DNA or RNA SIP, it requires about 30% of ^{13}C incorporation into the nucleic acids while the difference in the isotope contents of peptides can be detected with 1-2% ^{13}C incorporation (Dumont and Murrell 2005, Jehmlich *et al.* 2008). Lipid- SIP has higher sensitivity (detection limit with only < 0.1% incorporation), however, in comparison to lipid-SIP, proteins occupy an obvious closer linkage to the phylogenetical information (Jehmlich *et al.* 2008, Vogt et al. 2016). Protein-SIP is therefore regarded as a powerful tool for analyzing both specific metabolic activities and phylogenetic information of microbial communities. A systematic protein-SIP-approach to identify functional proteins expressed by microbial communities during the incubation of ^{13}C-labelled substrates has been described by Jehmlich and colleagues and is used in this thesis (Jehmlich et al. 2008, Jehmlich *et al.* 2008, Jehmlich *et al.* 2010).

1.4 Aims of the thesis

The general aim of the thesis is to characterize the (anaerobic) hydrocarbon and phenols degradation by using stable isotope tools. Within this scheme, several subprojects have been outlined:

1) The development of a valid method for carbon and hydrogen isotope analysis for phenols and to provide a first data set for isotope fractionation upon the initial steps of different aerobic and anaerobic phenol and cresols degradation pathways.

2) The development of a valid method for carbon and hydrogen isotope analysis for *n*-hexadecane analysis and to provide a first data set for isotope fractionation upon the initial steps of *n*-hexadecane degradation by *Desulfococcus oleovorans* strain Hxd3.

3) The identification of the “key-player(s)” of anaerobic ethylbenzene degradation in a sulfate reducing enrichment culture obtained from a contaminated aquifer in Zeitz, Germany.

4) The development of carbon and isotope analysis methods with lower detection limits for BTEX field sample analysis as well as the first insight into the ethylbenzene degradation in the field site Òdena.

2 MATERIALS AND METHODS

2.1 Materials and microorganisms

All chemicals and reagents used in this thesis, if not stated otherwise, were purchased from Alfa Aesar GmbH&Co.KG (Karlsruhe, Germany), AppliChem GmbH (Darmstadt, Germany), Merck KGaA (Darmstadt, Germany), Carl Roth GmbH & Co.KG (Karlsruhe, Germany) and Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

Acinetobacter calcoaceticus NCIMB 8250 and *Pseudomonas pseudoalcaligenes* NCIMB 9867 were purchased from the NCIMB culture collection (NCIMB Ltd, Aberdeen, Scotland). *Azoarcus buckelii* DSM 14744, *Desulfosarcina cetonica* DSM 7267 and *Thauera aromatica* DSM 6984 were obtained from the Leibnitz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *Desulfococcus oleovorans* Hxd3 was kindly provided by Prof. Dr. Johann Heider, University Marburg, Germany. *Geobacter metallireducens* DSM7210 adapted to growth on cresols under nitrate-reducing conditions was kindly provided by Prof. Dr. Matthias Boll, University Freiburg, Germany.

2.2 Microbiological methods

2.2.1 Aerobic and anaerobic cultivation techniques

The aerobic media were prepared in 1 L Schott bottles (DURAN ®), sealed with non-gas-tight caps (DURAN ®) and sterilized by autoclavation. The medium was aliquoted to serum bottles (120 mL/240 mL, Glasgerätebau Ochs Laborfachhandel e.K., Bovenden, Germany), inoculated and sealed with non-gas-tight Teflon-coated butyl septum (Glasgerätebau Ochs Laborfachhandel e.K., Bovenden, Germany). Samples were taken

through syringes with different sizes (Ominifix, B. Braun Melsungen Ag, Melsungen, Germany). The aliquotion, inoculation and sampling were performed using a sterile bench (Variolab Mobilien W90, WALDNER Laboreinrichtungen GmbH & Co. KG, Wangen, Germany).

The anoxic media for cultivation of anaerobic microorganism were prepared as follows: firstly, the basal medium was prepared and autoclaved in 1 L Schott bottles (DURAN ®) and the medium bottle was flushed with N_2 for 2 hours. Subsequently, the medium was sealed with sterilized butyl rubber stoppers (Glasgerätebau Ochs Laborfachhandel e.K., Bovenden, Germany). The anoxic sterilized basal medium was afterwards complemented with anoxic vitamin and anoxic trace element solution. For certain cultivation purpose, the medium was aliquoted to smaller serum bottles (120 mL/240 mL, Glasgerätebau Ochs Laborfachhandel e.K., Bovenden, Germany) and sealed with Teflon-coated butyl rubber stopper (Glasgerätebau Ochs Laborfachhandel e.K., Bovenden, Germany). Due to the sampling during the long cultivation process, medium loss was replenished by the addition of fresh medium. Medium complementation, the aliquotion, and the addition of fresh medium were carried out in an anaerobic glove box (gas atmosphere 97% N_2 and 3% H_2; Coy Laboratory Products Inc., Grass Lake, MI USA).

The anoxic solutions were firstly sterilized by either autoclaving or filtration and then oxygen was removed by flushing the solution with N_2 for a certain time according to the volume of the solution.

The inoculation of the anaerobic cultures were performed with syringes (different sizes, Ominifix, B. Braun Melsungen Ag, Melsungen, Germany) pre-flushed with N_2 on the sterile bench (Variolab Mobilien W90, WALDNER Laboreinrichtungen GmbH & Co. KG, Wangen, Germany). Samples were taken with syringes of different sizes (Ominifix, B. Braun Melsungen Ag, Melsungen, Germany) pre-flushed with N_2.

2.2.1.1 Cultivation of *A. calcoaceticus* NCIMB 8250 and *P. pseudoalcaligenes* NCIMB 9867

A.calcoaceticus NCIMB 8250 and *P. pseudoalcaligenes* NCIMB 9867 were cultivated under oxic condition in Brunner mineral salt medium which was composed of basal medium, vitamin solution and trace element solution according to Vogt and colleagues (Table 2.1). (Vogt et al. 2002) .

Table 2.1: Composition of Brunner mineral salt medium

Basic medium	
Component	Amount per liter
Na_2HPO_4	2.44 g
KH_2PO_4	1.52 g
$(NH_4)_2SO_4$	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$CaCl_2 \cdot 2H_2O$	0.05 g
Composition ratio	
Basic medium	980 mL
Trace element solution SL-10*	10 mL
Vitamin solution for Brunner medium*	10 mL

* The composition of trace element and vitamin solution is described separately in chapter 4.2.1.9.

The pH of the medium was adjusted to 7.0 with 2M HCl solution after the medium complementation. 135 mL of the media were aliquoted into 240 mL serum-bottles and 15 mL of bacterial pre-culture was inoculated into the medium. Abiotic control was set up with 150 mL same medium. Then different amounts of phenol or cresols stock solution were added with glass syringe (Hamilton, Reno, NV, USA) as shown in Table 2.2.

Table 2.2: Substrate concentrations used for *A.calcoaceticus* NCIMB 8250 and *P.pseudoalcaligenes* NCIMB 9867

	Concentration of stock solution	Volume added into the culture	Culture volume	Final concentration in culture
Phenol	0.5 M	300 µL	150 mL	1 mM
p-Cresol	0.1 M	1500 µL	150 mL	1 mM

The cultures were cultivated at 28 °C and 75 rpm in an incubator (ISF-1-W, Adolf Kühner AG, Birsfelden, Switzerland).

2.2.1.2 Cultivation of *A.buckelii* DSM 14744 and *T.aromatica* DSM 6984

A.buckelii DSM 14744 and *T. aromatica* DSM 6984 were cultivated under anoxic conditions in a modified DSMZ medium 586 (TA medium) composed of five solutions (Table 2.3).

Table 2.3: Composition of TA medium

TA solution A	
Component	Amount per liter
K_2HPO_4	5.92 g
KH_2PO_4	0.816 g
TA solution B	
$MgSO_4 \cdot 7H_2O$	26.4 g
$CaCl_2 \cdot 2H_2O$	3.2 g
NH_4Cl	70 g
Composition ratio	
TA solution A	960 mL
TA solution B	6 mL
Trace element solution SL-10*	10 mL
Vitamine solution for TA medium*	5 mL
KNO_3 stock solution*	20 mL

* The composition of trace element, vitamin and stock solution is described separately in chapter 2.2.1.9.

The pH of the medium was adjusted to 7.0 with 2 M HCl solution. 135 mL of the media were poured into each 240 mL serumbottles and 15mL of bacterial pre-culture was inoculated into the medium. Abiotic control was set up with 150 mL same medium. Then different amounts of phenol or cresols stock solution were added as shown in Table 2.4.

Table 2.4: Substrate concentration used for *A.buckelii* DSM 14744

	Concentration of stock solution	Volume added into the culture	Culture volume	Final concentration in culture
p-Cresol	0.1 M	1500 µL	150 mL	1 mM

The cultures were cultivated at 28 °C and 75 rpm in an incubator (ISF-1-W, Adolf Kühner AG, Birsfelden, Switzerland).

2.2.1.3 Cultivation of *Desulfosarcina cetonica* DSM 7267

D.cetonica DSM 7267 was cultivated under anoxic conditions in a mineral salt medium described by Vogt and colleagues (Vogt et al. 2007) with modifications (Table 2.5).

Table 2.5: Composition of mineral salt medium for *D. cetonica* DSM 7267

Basic medium	
Component	Amount per liter
NaCl	10 g
KH_2PO_4	0.5 g
NH_4Cl	0.4 g
KCl	0.4 g
Na_2SO_4	0.2 g
$CaCl_2$	0.1 g
$MgCl_2$	0.5 g
Composition ratio	
Basic medium	945 mL
$NaHCO_3$ stock solution*	30 mL
Na_2SO_4 stock solution*	20 mL
Trace element solution SL-10*	1 mL
Vitamin solution for mineral salt medium*	5 mL
Selenite-tungsten solution*	1 mL

* The composition of trace element, vitamin, selenite-tungsten and stock solutions are described separately in chapter 2.2.1.9.

The pH of the medium was adjusted to 7.0 with 2M HCl solution. 135 mL of the media were poured into each 240 mL serum-flasks and 15 mL of bacterial pre-culture grown on 0.5 M toluene was inoculated into the medium. Abiotic control was set up with 150 mL same medium without inoculum. Then different amounts of phenol or cresols stock solution were added as shown in Table 2.6.

Table 2.6: Substrates concentration used for *D.cetonica* DSM 7267

	Concentration of stock solution	Volume added into the culture	Culture volume	Final concentration in culture
Phenol	0.5 M	150 µL	150 mL	0.5 mM

p-Cresol	0.1 M	750 µL	150 mL	0.5 mM
m-Cresol	0.1 M	750 µL	150 mL	0.5 mM

The cultures were cultivated at 18 °C and 150 rpm on an incubator (Bühler Labtec GmbH, SM-25 Control).

2.2.1.4 Cultivation of *D. oleovorans* strain Hxd3

D.oleovorans strain Hxd3 was cultivated under anoxic condition in a medium described by Aeckersberg and colleagues (Aeckersberg *et al.* 1991). The composition of the medium is shown in Table 2.7.

Table 2.7: Composition of mineral salt medium for *D.oleovorans* strain Hxd3

Basic medium	
Component	Amount per liter
NaCl	20 g
KH_2PO_4	0.2 g
NH_4Cl	0.25 g
KCl	0.50 g
Na_2SO_4	4 g
$CaCl_2 \cdot 2H_2O$	0.15 g
$MgCl_2 \cdot 6H_2O$	3 g
Resazurin	1 mg
Composition ratio	
Basic medium	958.9 mL
$NaHCO_3$ stock solution*	30 mL
$Na_2S \cdot 9H_2O$ stock solution*	7.5 mL
Trace element solution SL-10*	1 mL
Vitamin solution*	1 mL
Selenite-tungsten solution*	1 mL
$Na_2S_2O_4$ stock solution*	600 µL

* The composition of trace element, vitamin, selenite-tungsten and stock solutions are described separately in chapter 2.2.1.9.

The pH of the medium was adjusted to 7.0 with 2M HCl solution. 70 mL of the media were poured into each 120 mL serum-flasks and 10 mL of pre-culture grown on *n*-

hexadecane was transferred to the medium. Abiotic control was set up with 80 mL same medium without inoculum. Then an amount of 29.4 µL *n*-hexadecane was injected into each culture with a glass syringe (Hamilton, Reno, NV, USA) to obtain a final substrate concentration of 1.25 mM. The cultures were cultivated at 28 °C and 75 rpm in an incubator (ISF-1-W, Adolf Kühner AG, Birsfelden, Switzerland).

2.2.1.5 Cultivation of *G.metallireducens* DSM 7210

G.metallireducens DSM 7210 was cultivated under anoxic condition in a medium described by Lovely and colleagues (Lovley and Phillips 1988) with modifications. The composition of the medium is show in Table 2.8.

Table 2.8: Composition of mineral salt medium for *G.metallireducens* DSM 7210

Basic medium	
Component	Amount per liter
NaCl	0.1 g
$NaH_2PO_4 \cdot H_2O$	0.6 g
NH_4Cl	1.5 g
KCl	0.1 g
$MgSO_4 \cdot 7H_2O$	0.1 g
$MnCl_2 \cdot 4H_2O$	5 mg
$MgCl_2 \cdot 6H_2O$	0.1 g
$NaMoO_4 \cdot 2H_2O$	1 mg
Composition ratio	
Basic medium	945 mL
$NaHCO_3$ stock solution*	5 mL
$NaNO_3$ stock solution*	10 mL
Trace element solution SL-10*	1 mL
Vitamin solution for mineral salt medium*	1 mL
Selenite-tungsten solution for mineral salt medium*	1 mL
Sodium ascorbate stock solution*	1 mL

* The composition of trace element, vitamin, selenite-tungsten and stock solutions are described separately in chapter 2.2.1.9.

The pH of the medium was adjusted to 7.0 with 1M NaOH solution. 135 mL of the media were poured into each 240 mL serum-flasks and 15 mL of bacterial pre-culture was

transferred to the medium. Abiotic control was set up with 150 mL same medium without inoculum. Then different amounts of phenol or cresols stock solution were added as shown in Table 2.9.

Table 2.9: Substrates concentration used for *G.metallireducens* DSM 7210

	Concentration of stock solution	Volume added into the culture	Culture volume	Final concentration in culture
p-Cresol	0.1 M	750 µL	150 mL	0.5 mM

The cultures were cultivated at 28 °C and 75 rpm in an incubator (ISF-1-W, Adolf Kühner AG, Birsfelden, Switzerland).

2.2.1.6 Cultivation of ethylbenzene-degrading enrichment culture from Zeitz under sulfate-reducing and nitrate-reducing conditions

For the setting up of microcosms, sand granules from Zeitz columns system (Vogt *et al.* 2007) were transferred into 1 liter Duran® Schott bottles which were filled immediately with anoxic groundwater and closed with butyl stoppers and screw caps. The groundwater was exchanged inside an anaerobic glove box with anoxic mineral salt medium containing 20 mM sulfate or nitrate as terminal electron acceptors ((Vogt *et al.* 2007), Table 2.10).

Table 2.10: Composition of mineral salt medium for Zeitz enrichment cultures

Basic medium	
Component	Amount per liter
NaCl	0.5 g
KH_2PO_4	0.5 g
NH_4Cl	0.4 g
KCl	0.4 g
Na_2SO_4	0.2 g
$CaCl_2$	0.1 g
$MgCl_2$	0.5 g
Composition ratio	
Basic medium	945 mL

$NaHCO_3$ stock solution*	30 mL
$Na_2SO_4/NaNO_3$ stock solution*	20 mL
Trace element solution SL-10*	1 mL
Vitamin solution for mineral salt medium*	5 mL
Selenite-tungsten solution for mineral salt medium*	1 mL

* The composition of trace element, vitamin, selenite-tungsten and stock solutions are described separately in chapter 2.2.1.9.

The pH of the medium was adjusted to 7.0 with 2M HCl solution. 61 µL ethylbenzene was injected into the culture by a glass syringe (Hamilton, Reno, NV, USA) to reach a final substrate concentration of 0.5 mM. The cultures were incubated in dark and room temperature.

2.2.1.7 Cultivation of ethylbenzene-degrading enrichment culture from Òdena, Barcelona, Spain under sulfate-reducing, nitrate-reducing or iron-reducing conditions

Field sampling methods are described in chapter 2.6.2. For the setting up of the microcosms, the sampled sludge was flushed with N_2 for 1.5 hours to remove any volatile contaminants inside. 85 mL medium was filled into each 120 mL serum bottle with either 20 mM sulfate, 20 mM nitrate or 10 mM Fe^{3+} (Table 4.11) as terminal electron acceptors. Afterwards, 15 mL sludge was inoculated into each culture. Duplicates were set up for samples from each well and under each condition. Abiotic controls were set up with 100 mL of the same medium.. No-substrate controls were set up the same with enrichment cultures but without addition of ethylbenzene. Subsequently, the culture flasks were sealed with Teflon septum and aluminum caps. 6.1 µL ethylbenzene was injected into each culture by a glass syringe (Hamilton, Reno, NV, USA) to reach a final substrate concentration of 0.5 mM. The cultures were incubated in an incubator (ISF-1-W, Adolf Kühner AG, Birsfelden, Switzerland) at room temperature and 85 rpm.

Table 2.11: Terminal electron acceptor in media for sulfate-, nitrate- and iron-reducing conditions

	Concentration of stock solution	Volume added into the medium	Medium volume	Final concentration
$Fe(OH)_3$ *	0.1 M	100 mL	1 L	10 mM
Na_2SO_4	1 M	20 mL	1 L	20 mM

$NaNO_3$	1 M	20 mL	1 L	20 mM

* $Fe(OH)_3$ was lab stock prepared by Dr.Steffen Kümmel in the same lab (Kümmel, personal communication) using the method described by Lovley and colleagues (Lovley 2013) .

2.2.1.8 Cultivation of stable isotope probing cultures using ring-labeled ethylbenzene as substrate under sulfate-reducing condition

The stable isotope probing cultures were set up in an anaerobic glove box. 50 mL active sand was aliquoted from one of the enrichment ethylbenzene-degrading cultures under sulfate-reducing condition and filled into a 120 mL serum bottle. 50 mL mineral salt medium with 20 mM sulfate as terminal electron acceptor (described in chapter 2.2.7) was added afterwards. Fourteen bottles of microcosms were set up in total. Abiotic controls were set up with 50 mL same medium and triply autoclaved active sand (121°C, 20 min) No-substrate controls were set up the same with enrichment cultures but without addition of ethylbenzene. Seven microcosms and one abiotic control were injected with 3.05 µL ethylbenzene to reach a final substrate concentration of 0.5 mM. While seven microcosms and one abiotic control was injected with 3.28 µL ring-labeled ethylbenzene (synthesized by the working group of Prof.Dr.Christoph Schneider, Institute of Organic Chemistry, University of Leipzig) to reach a labeled substrate concentration of 0.5 mM. The cultures were incubated in dark at room temperature in an incubator at 150 rpm (Bühler Labtec GmbH, SM-25 Control).

2.2.1.9 Composition of stock solutions used in the cultivation media

Table 2.12 Trace element solution SL-10 (1 L)

HCl (25%; 7.7 M)	10 mL
$FeCl_2 \cdot 4H_2O$	1.5 g
$ZnCl_2$	70 mg
$MnCl_2 \cdot 4H_2O$	100 mg
H_3BO_3	6 mg
$CoCl_2 \cdot 6H_2O$	190 mg
$CuCl_2 \cdot 2H_2O$	2 mg
$NiCl_2 \cdot 6H_2O$	24 mg
$Na_2MoO_4 \cdot 2H_2O$	36 mg
Milli-Q® water	approximately 990 mL

Table 2.13 Vitamin stock solution for Brunner medium (1 L)

p-Aminobenzoate	10 mg
Biotin	2 mg
Nicotinic acid	20 mg
Thiamine-HCl·$2H_2O$	10 mg
Ca-pantothenate	5 mg
Pyridoxamine	50 mg
Vitamin B_{12}	20 mg
Milli-Q® water	approximately 1000 mL

Table 2.14 Vitamin stock solution for TA medium (1 L)

p-Aminobenzoate	50 mg
Biotin	20 mg
Nicotinic acid	25 mg
Thiamine-HCl·$2H_2O$	50 mg
Pantothenic acid	50 mg
Pyridoxamine-HCl	10 mg
Vitamin B_{12}	50 mg
Nicotin amide	25 mg
Riboflavin	50 mg
Folic acid	20 mg
α- Lipoic acid	50 mg
Milli-Q® water	approximately 1000 mL

Table 2.15 Vitamin solution for mineral salt medium (1 L) (Vogt *et al.* 2007)

p-Aminobenzoic acid	8 mg
Biotin	2 mg
Nicotinic acid	20 mg
Thiamine-HCl	20 mg
Pantothenate	10 mg
Pyridoxamine-HCl	30 mg
Milli-Q® water	approximately 1000 mL

Table 2.16 Vitamin stock solution for *D.oleovorans* strain Hxd3 medium (1 L)

p-Aminobenzoic acid	80 mg
Biotin	20 mg
Nicotinic acid	200 mg
Thiamine-HCl	200 mg
Pantothenate	100 mg
Pyridoxamine-HCl	300 mg
Cyanocobalamin	100 mg
Milli-Q® water	approximately 1000 mL

Table 2.17 KNO_3 stock solution for TA medium (1 L)

KNO_3	100.8 g
Milli-Q® water	approximately 900 mL

Table 2.18 Selenite-tungsten solution for mineral salt medium (1 L)

NaOH	0.5 g
$Na_2SeO_3 \cdot 5H_2O$	3 mg
$Na_2WO_4 \cdot 2H_2O$	4 mg
Milli-Q® water	approximately 1000 mL

Table 2.19 Selenite-tungsten solution for *D.oleovorans* strain Hxd3 medium (1 L)

NaOH	0.4 g *D.oleovorans* strain Hxd3 medium
$Na_2SeO_3 \cdot 5H_2O$	6 mg
$Na_2WO_4 \cdot 2H_2O$	8 mg
Milli-Q® water	approximately 1000 mL

Table 2.20 $NaHCO_3$ stock solution (1 L)

$NaHCO_3$	84 g
Milli-Q® water	approximately 950 mL

Table 2.21 Na_2SO_4 stock solution (1 L)

Na_2SO_4	142 g
Milli-Q® water	approximately 900 mL

Table 2.22 Na_2S stock solution *D.oleovorans* strain Hxd3 medium (1 L)

$Na_2S \cdot 9H_2O$	48 g
Milli-Q® water	approximately 970 mL

Table 2.23 $Na_2S_2O_4$ stock solution for *D.oleovorans* strain Hxd3 medium (1 L prepared freshly)

$Na_2S_2O_4$	50 g
Milli-Q® water	approximately 960 mL

Table 2.24 $NaNO_3$ stock solution (1 L)

$NaNO_3$	85.00 g
Milli-Q® water	approximately 950 mL

Table 2.25 Sodium ascorbate stock solution for *G.metallireducens* DSM 7210 medium (1 L)

$C_6H_7NaO_6$	39.62 g
Milli-Q® water	approximately 970 mL

2.2.1.10 Storage of bacterial cultures

Short-term preservation of cultures took place at 4 °C in the dark. For the purpose of long-term preservation, the cultures were transferred to fresh medium every month.

2.3 Analytical methods

2.3.1 Determination of growth

The optical density of *A. calcoaceticus* NCIMB 8250 and *P. pseudoalcaligenes* NCIMB 9867 was determined in transparent plastic cuvettes at a wavelength of 578 nm by a

spectrophotometer (Pharmacia Biotech Novaspec II, Uppsala Sweden). An abiotic control containing medium and substrate was used as sample blank.

2.3.2 Analysis of substrates and products

2.3.2.1 Analysis of BTEX (benzene, toluene, ethylbenzene, xylene)

Gas chromatography was used to measure the concentration of ethylbenzene and other BTEX compounds in cultures. The samples were analyzed by automated headspace analysis with a Varian 3800 gas chromatograph (Varian, USA) packed with a CP SIL 5 CB capillary column (25 m x0.12 mm ID x 0.12 µm FD; Varian, Germany) and a flame ionization detector. The analytical program used was as follows: injector temperature, 250 °C (split 1:50); detector temperature, 260 °C; and an oven temperature program holding of 70 °C for 2 min, followed by an increase at a rate of 10 °C/min till 90 °C and the at a rate of 60 °C/min till 220 °C. The carrier gas was molecular nitrogen in a flow of 1 mL/ min. Samples were diluted by 1:20 using 1.6 mM H_2SO_4 with a final volume of 10 mL. The samples were prepared in 20 mL glass vials (Ochs Laborbedarf, Bovenden, Germany) and closed with gas-tight butyl septa crimped by aluminium caps (Axel Semrau GmbH/Co.KG, Sprockhövel, Germany). The samples were incubated at 70 °C for 30 min in an agitator prior to analysis and 1 mL of headspace was injected. For calibration, diluted standards of BTEX were treated in the same way as the samples.

2.3.2.2 Analysis of phenol and cresols

The concentration of phenol and cresols were analyzed by HPLC or GC. This was determined by the methods used for subsequent carbon or hydrogen isotope analysis: carbon stable isotopes were analyzed using LC-IRMS and hydrogen isotopes were analyzed using GC-IRMS. The same samples taken at each time point were used for both concentration and isotope analysis to avoid possible mistakes by sampling.

In studies linked to phenol and cresols carbon isotope analysis, the concentration of phenol and cresols were analyzed by HPLC using a Shimadzu Prominence instrument (Shimadzu Deutschland GmbH, Duisburg, Germany). Samples for HPLC analysis were filtered (Whatmann PuradiscTM 0.45 µm PES Filter) and subsequently acidified to pH 2 with 6 M H_2SO_4; if not analyzed immediately, the samples were stored at -20 °C. Ten µL sample was injected by an autosampler (SIL-20A, Shimadzu Deutschland GmbH, Duisburg, Germany). A reverse phase column LiChrospher® 100 RP-18 (Merck KGaA,

Darmstadt, Germany; 5 µm particle size, 4 mm (column diameter) x 250 mm (column length)), was used as stationary phase. Methanol (HPLC grade > 99.9%) and 0.1% phosphoric acid were used as mobile phase. During measurement, the mix ratio of the two solutions was 1:1 and the flow rate of the mobile phase was held constantly at 0.5 mL/min for 12 minutes. The oven temperature was kept constant at 25 °C.

In studies linked to phenol and cresols hydrogen isotope analysis, the concentration of phenol and cresols were analyzed by a GC (7890A, Agilent Technologies, Santa Clara, CA, USA) coupled to a MS (5975C, Agilent Technologies, Santa Clara, CA, USA). A ZB-1 column (60m length x 0.32 mm ID, 1 µm film thickness; J&W Scientific, Agilent Technologies, USA) was used for separation. 200 mL cultures were acidified to pH 2 with 6 M H_2SO_4 and subsequently extracted with 4 mL benzene by gently shaking at 4 °C for 48 hours. The extracts were used for both concentration determination and hydrogen isotope analysis. The samples for concentration analysis were prepared in 2 mL vials (2 mL screw cap vials, 12x32 mm, WICOM Germany GmbH, Heppenheim, Germany) with micro inserts (100 µL, 5 mm AD, WICOM Germany GmbH, Heppenheim, Germany) and 1 µL was injected to the system by an auto sampler (CTC, Zwingen, Switzerland) in split mode (split ratio of 1:20). The injector temperature was 250 °C and helium was used as the carrier gas at a constant rate of 1.5 mL/min. An oven temperature program holding of 45 °C for 5 min, followed by an increase at a rate of 5 °C/min till 120 °C and then at a rate of 20 °C/min till 250 °C was used for separation. For calibration, standards solutions were treated in the same way as samples.

2.3.2.3 Analysis of *n*-alkanes

The concentrations of *n*-alkanes were determined by a gas chromatograph equipped with a flame ionization detector (GC-FID, 7820A, Agilent Technologies, USA). A HP-5 column (30m length x 0.25 mm ID, 0.25 µm film thickness; J&W Scientific, Agilent Technologies, USA) was used for separation. Each 150 mL culture was extracted with 3 mL *n*-hexane by gently shaking at 4 °C for 48 hours. The samples for concentration analysis were prepared in 2 mL vials (2 mL screw cap vials, 12x32 mm, WICOM Germany GmbH, Heppenheim, Germany) with micro inserts (100 µL, 5 mm AD, WICOM Germany GmbH, Heppenheim, Germany) and 1.5 µL was injected to the system by an auto sampler (CTC, Zwingen, Switzerland) in split mode (split ratio of 1:5). Both the injector and detector temperature was held at 250 °C and helium was used as the

carrier gas at a constant rate of 1.5 mL/min. An oven temperature program holding of 45 °C for 4 min, followed by an increase at a rate of 1 °C/min till 50 °C and then at a rate of 12 °C/min till 220 °C and was held furthermore for 1 min. For calibration, standards solutions were treated in the same way as samples.

2.3.2.4 Analysis of hydrogen sulfide

Sulfide was determined photometrically by a method described originally by Cline (1969) using modifications described by Herrmann and colleagues (2008). Immediately after sampling, the sample (0.5 mL) was added to 1 mL zinc acetate dehydrate solution (20 g/L) for sulfide fixation. Afterwards, 4 mL distilled water and 400 µL Cline reagent (*N,N*-dimethyl-*p*-phenylendiammoniumdichloride) were added. The reaction mixture was vortexed (Vortex Genie-2, Model G-560E, Scientific Industries, INC, BOHEMIA, N.Y, 11716, USA) and kept in the dark for 20 minutes. Subsequently, the absorption of the reaction mixture was measured at 670 nm with a spectrometer (Novaspec II, Pharmacia AG, Uppsala, Sweden). Distilled water was used as sample blank.

2.3.2.5 Analysis of nitrite

Nitrite was determined by a modified Griess reaction in accordance with the German standard DIN EN 26 777. Dilution was performed with distilled water according to the possible nitrite concentration in the sample. Subsequently, 250 µL Griess reagent (phosphoric acid, sulfanilamide, and *N*-1-napthylenediamine dihydrochloride) was added to the sample or diluted sample. The reaction mixture was stored in the dark for 10 minutes and the absorption was then measured at 540 nm. Distilled water was used as sample blank.

2.3.2.6 Analysis of metabolites

Putative metabolites due to ethylbenzene degradation in the Zeitz enrichment cultures were analyzed by a GC (7890A, Agilent Technologies, Santa Clara, CA, USA) coupled to a MS (5975C, Agilent Technologies, Santa Clara, CA, USA). For the analysis of metabolites, a derivatization step was performed in advance. The cultures were acidified to pH 2 with 6 M H_2SO_4 and extracted three times with 3 mL diethyl ether (anhydrous, ⩾ 99.7%) by shaking gently at 4 °C for 30 min. The solvent phases were combined and evaporated under a continuous nitrogen stream. The residual fraction was dissolved in 450 µL methanol (HPLC grade > 99.9%) followed by the addition of 50 µL

trimethylchlorosilane. The mixture was incubated at 65 °C for 90 min. Afterwards, the remaining solvent and reagent were removed under a continuous nitrogen flow. The obtained carboxylic acid methyl esters were dissolved in 100 µL *n*-hexane. The standard solution of 2-(1-phenylethyl)-succinic acid (synthesized by Working Group Professor Golding, School of Chemistry, Newcastle University) was prepared as follows: 5 mg of 2-(1- phenylethyl)- succinic acid was dissolved in 10 mL methanol to obtain a 500 µg/mL stock solution; subsequently, 100 µL stock solution was dissolved in 100 mL Milli-Q® water. The standard solution was treated using the same derivatization procedure as the cultures. The injector temperature was 250 °C and helium was used as the carrier gas at a constant rate of 1.5 mL/min. A Zebron BPX- 5 column (30m length x 0.25 mm ID, 0.25 µm film thickness, Phenomenex, Torrance, USA) was used for separation. The oven temperature program was held at 40 °C for 5 min, followed by an increase at a rate of 20 °C/min till 150 °C and at a rate of 2 °C/min till 250 °C and then at a rate of 20 °C/min till 300 °C and in the end holding of 300 °C for 2 min. The identification of 2- (1-phenylethyl) - succinic acid as metabolites in cultures was done by comparing to the mass spectrum of the standard solution.

2.3.3 Stable isotope analysis

2.3.3.1 Analysis of the enrichment of ^{13}C in headspace CO_2

The mineralization of ring labeled ^{13}C-ethylbenzene was proved by detection of the accumulation of $^{13}CO_2$ in the gas phase of the cultures. The carbon isotope composition of CO_2 was determined by a gas chromatograph (7890A series, Agilent Technologies, Santa Clara, CA, USA) coupled to an isotope mass spectrometer (MAT 253, Thermo Finnigan, Bremen, Germany) via a ConFlo IV interface (Thermo Finnigan, Bremen, Germany). The combustion furnace was held at 1000 °C on a Cu/Ni catalyst. The separation was performed by a PoraBond Q column (50m x 0.32 mm, 1 µm, Agilent Technologies, Santa Clara, CA, USA). The oven temperature was held isothermally at 40 °C using nitrogen as carrier gas at a flow of 2.0 mL/ min. The injector temperature was held at 250 °C and samples were injected manually at splitless mode or 1:5 to 1: 100 split ratios. The carbon isotope signatures were obtained as δ notation (per mil) relative to the Vienna Pee Dee Belemnite standard (V-PDB). For analysis, 5 mL glass vials (Ochs Laborbedarf, Bovenden, Germany) were flushed by continuous helium flow for 1 min and closed immediately with gas-tight Teflon septa and aluminum caps (Ochs Laborbedarf,

Bovenden, Germany). 1 mL gas phase of the culture was taken and injected into the vials. Samples of 100 µL to 1000 µL from these vials were injected for analysis by using glass syringes (Hamilton, Reno, NV, USA).

2.3.3.2 Carbon and hydrogen isotope analysis of ethylbenzene

Liquid samples for ethylbenzene isotope analysis were taken from the cultures with a volume of 15 mL and were filled into 20 mL vials (Ochs Laborbedarf, Bovenden, Germany). For those samples at very late time points of a degradation experiments, 30 mL of samples were taken because of the low remaining substrate concentration, and were filled into 50 mL vials (Ochs Laborbedarf, Bovenden, Germany). The samples were conserved immediately with 200 µL H_2SO_4 (6 M) and closed with Teflon coated screw caps (WICOM Germany GmbH, Heppenheim, Germany). Ethylbenzene was extracted with 1 mL *n*-pentane by gently shaking at 4 °C for 48 hours. Carbon isotope signatures of ethylbenzene were analyzed by a gas chromatograph (6890 series, Agilent Technologies, Santa Clara, CA, USA) coupled to an isotope mass spectrometer (MAT 252, Thermo Finnigan, Bremen, Germany) via a ConFlo III interface (Thermo Finnigan, Bremen, Germany). The combustion furnace was held at 940 °C on a Cu/Ni/Pt catalyst. The analytes were separated by using a BPX-5 column (30 m length x 0.25 mm ID, 0.25 µm film thickness, Phenomenex, Torrance, USA). The temperature of the GC oven was held at 40 °C for 6 min, was then increased at a rate of 3 °C/ min to 90 °C and was increased finally at a rate of 20 °C/ min to 300 °C which was held for 5 min. Stable hydrogen isotopes of ethylbenzene were analyzed by gas chromatography pyrolysis isotope ratio mass spectrometry (GC-P-IRMS, GC, 7890A series, Agilent Technologies, Santa Clara, CA, USA; IRMS, Thermo Finnigan, Bremen, Germany). The organically bound hydrogen was converted to H_2 by a high temperature pyrolysis at 1420 °C in non-porous alumina tube reactors. The sampling and extraction method, the GC column and oven temperature program was the same as described above for carbon isotope analysis.

2.3.3.3 Hydrogen stable isotope analysis of BTEX by large volume injection

The method for hydrogen stable isotope analysis of BTEX by large volume injection was developed based on a method described elsewhere (Herrero-Martin *et al.* 2015) with modifications. Thirteen milliliter samples were taken from each Òdena underground water samples or standards and filled into 20 mL headspace vials containing 5 g NaCl and

then closed immediately with magnetic caps with PTFE septa (Th. Geyer, Renningen, Germany). The samples were incubated at 90 °C, 500 rpm for 10 min. Afterwards, 5 mL headspace samples were taken with a pre-heated syringe (temperature 70 °C) and injected into a Multimode Inlet (MMI) Programmed Temperature Vaporizer (PTV, G35110A, Agilent Technologies, Waldbronn, Germany) under "Solvent Vent Mode". The condition of PTV was programmed as follows: the split valve was opened and the headspace sample was introduced into the injector which was pre-cooled to 0 °C by liquid nitrogen at a vent flow of 5 mL/ min and a vent pressure of 5 psi; the split valve was then closed for 30 s while the liner temperature was increased at a rate of 650 °C/ min to 300 °C for the release of the target compounds; 2.9 min after the injection, the split valve was opened again and the liner temperature was held isothermally at 300 °C for 10 min. The isotope signatures were determined by a gas chromatograph (7890A series, Agilent Technologies, Santa Clara, CA, USA) coupled to an isotope mass spectrometer (MAT 253, Thermo Finnigan, Bremen, Germany) via a ConFlo IV interface (Thermo Finnigan, Bremen, Germany). The organically bound hydrogen was converted to H_2 by a high temperature pyrolysis at 1420 °C in non-porous alumina tube reactors. The analytes were separated by using a BPX-5 column (30 m length x 0.25 mm ID, 0.25 µm film thickness, Phenomenex, Torrance, USA). The temperature of the GC oven was held at 35 °C for 10 min, was then increased at a rate of 4 °C/ min to 150 °C and was increased finally at a rate of 20 °C/ min to 260 °C which was held for 5 min.

2.3.3.4 Carbon stable isotope analyses of phenol and cresols by LC-IRMS

Carbon isotope signatures of phenol and cresols were determined by a high pressure liquid chromatograph coupled via LC-isolink to a Finnigan MAT 253 isotope ratio mass spectrometer (HPLC-IRMS, Thermo Fisher Scientific, Bremen, Germany). The HPLC system was equipped with a HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland), a Surveyor MS Pump Plus (Thermo Scientific, Bremen, Germany) and a Jetstream 2 Plus column thermostat (Sigma-Aldrich, Steinhagen, Germany). Compounds were separated on an YMC Triart column (75 mm, length x 2 mm, diameter, 3 µm particles size). Milli-Q water was used as the eluent and was pumped with a flow rate of 400 µL/ min. The analytes were oxidized through a wet oxidation with phosphoric acid (147 g/ L) and sodium peroxodisulfate (200 g/ L). The flow rates of both reagents for oxidation reactions were set to 50 µL/ min. Samples were prepared as described in chapter 2.3.2.2. The injection volume was from 25 to 100 µL of sample. Standards of

phenol and cresols dissolved in the eluent were regularly analyzed to ensure the data quality. Standards of phenol and *m*-cresol, *o*-cresol, *p*-cresol prepared in the eluent were analyzed after every ten samples to ensure data quality of $\delta^{13}C$ values.

2.3.3.5 Hydrogen stable isotope analysis of phenols by GC-Cr/HTC-IRMS

For the analysis of phenols hydrogen isotope signatures, a derivatization step was performed before isotope measurement. Seven compounds, phenol, *m*-cresol, *p*-cresol, *o*-cresol, 3,5-dimethylphenol, 2,4-dimethylphenol and 2,5-dimethylphenol were used as standards for method development. The standards solution with a series of concentration were prepared using benzene as solvent in 5 mL reaction vials (Tread bottle, WICOM Germany GmbH, Heppenheim, Germany) as shown in Table 4.26. The phenols stock solutions were prepared in a concentration of 10 mg/mL. Trifluoroacetic anhydride (TFAA) was used as derivatization reagent and was added to each standard solution according to the amount shown in Table 4.26 (Reaction mechanism is described in chapter 3.3.1.3). The corresponding molar concentration of the standards is shown in Table 2.11. Afterwards, the reaction mix was vortexed (Vortex Genie-2, Model G-560E, Scientific Industries, INC, BOHEMIA, New York, USA) and incubated in the dark in an incubator (ISF-1-W, Adolf Kühner AG, Birsfelden, Schweiz) at 18 °C and 85 rpm overnight. The reaction vials were placed on ice and 1 mL NaOH solution (1.2%) was added into each vial to neutralize the acid produced during the derivatization reaction. Subsequently the mixture was vortexed again and incubated at 4 °C for 30 min. Finally, the benzene layer was taken by glass pipettes into 2 mL vials (2 mL Screw cap, 12x32 mm, WICOM Germany GmbH, Heppenheim, Germany) with micro inserts (100 µL, 5 mm AD, WICOM Germany GmbH, Heppenheim, Germany) for the subsequent analysis. The derivatization products were analyzed by a GC (7890A, Agilent Technologies, Santa Clara, CA, USA) coupled to a MS (5975C, Agilent Technologies, Santa Clara, CA, USA) equipped with a ZB-1 column (60m length x 0.32 mm ID, 1 µm film thickness; J&W Scientific, Agilent Technologies, USA). The injector temperature was 250 °C and helium was used as the carrier gas at a constant rate of 1.5 mL/min. The temperature of the GC oven was held isothermally at 45 °C for 5 minand subsequently increased at a rate of 2 °C/min to 160 °C, then at a rate of 20 °C/min to 320 °C. Analyses were performed in full scan mode with a scanning range from 50 to 600. Two hundred mL culture samples were firstly extracted with 4 mL benzene by gently shaking for 48 hours at 4 °C. The benzene extracts were subsequently derivatized with the same procedures described for

the standard solutions. The identification of derivatives in cultures was done by comparing to the mass spectrum of the standard solutions.

Table 2.26 Series standard solutions of phenols

Std. Number	Concentration (µg/mL)	Volume $_{\text{phenols stock solution}}$ (µL)	Volume $_{\text{pure benzene}}$ (µL)	Volume $_{\text{TFAA}}$ (µL)
1	10,000	500	0	180
2	5,000	250	250	125
3	2,000	100	400	50
4	1,000	50	450	30
5	800	40	460	30
6	500	25	475	20
7	300	15	485	20

Table 2.27 Molar concentrations of standards solutions

Std. Number	Mass concentration (µg/mL)	Molar concentration of phenol (mM)	Molar concentration of cresols (mM)	Molar concentration of dimethylphenols (mM)
1	10,000	106.4	92.6	82.6
2	5,000	53.2	46.3	41.3
3	2,000	21.3	18.5	16.5
4	1,000	10.6	9.3	8.3
5	800	8.5	7.4	6.6
6	500	5.3	4.6	4.1
7	300	3.2	2.8	2.5

The hydrogen stable isotope analysis of phenols was realized via a gas chromatograph-chromium-based high-temperature conversion- isotope ratio mass spectrometer (GC-Cr/HTC-IRMS, Thermo Finningan, Bremen, Germany). The reactor design of the chromium based GC-Cr/HTC-IRMS and validation is described by Renpenning and colleagues (Renpenning *et al.* 2015). The chromium-based reactor (Cr-HTC) was composed of a ceramic tube (240 mm, length x 0.8 mm, inner diameter, Degussit AL23 aluminum oxide ceramic, Friatec, Germany) filled with chromium powder (> 99%, particle size 250-300 µm, Cr Patinal, Merck, Germany) and 10 mm plugs of quartz wool (HEKAtech GmbH, Germany) at both ends. The reactor temperature was held at 1200 °C, and helium in a flow rate of 2 mL/ min was used as carried gas. One to five µL was injected at splitless mode or a split ratio of 1:5. The injector temperature was held at 250 °C. The oven temperature was held at 45 °C for 5 min, subsequently increased at a rate of 2 °C/min to 120 °C, followed by an increase of 20 °C/min to 250 °C. An *n*-alkane mix which contained *n*-dodecane, *n*-tetradecane, *n*-pentadecane, *n*-hexadecane and *n*-

octadecane was used as standard for calibration to correct any possible bias caused by the system.

2.3.3.6 Carbon and hydrogen isotope analysis of *n*-hexadecane

Carbon stable isotopes of *n*- hexadecane were analyzed using a gas chromatograph (7890A series, Agilent Technologies, Santa Clara, CA, USA) coupled to an isotope mass spectrometer (MAT 253, Thermo Finnigan, Bremen, Germany) via a ConFlo IV interface (Thermo Finnigan, Bremen, Germany). The combustion furnace was held at 1030 °C on a Cu/Ni catalyst. 80 mL bacterial culture was sacrificed by the addition of 6 M H_2SO_4 to a pH around 2 and extracted with 2 mL *n*-hexane by shaking gently at 4 °C for 48 hours. Standards of *n*-hexadecane with a concentration series of 2 mM, 1.5 mM, 1 mM, 0.5 mM, 0.3 mM, 0.1 mM, 0.05 mM, 0.03 mM and 0.01 mM were prepared in *n*-hexane. A laboratory standard of a BTEX mix (for carbon isotope analysis: 6 µL of each compound in 10 mL *n*-pentane), which was calibrated using the international standard, was analyzed before measurement and in between the measurement to ensure data quality of isotope values. One to five µL samples were injected to an injector held isothermally at 250 °C. A BPX-5 column (30 m length x 0.25 mm ID, 0.25 µm film thickness, Phenomenex, Torrance, USA) was used for separation. The GC oven temperature was firstly held at 45 °C for 4 min, subsequently increased at a rate of 1 °C/ min to 50 °C, followed by an increase ofthe rate of 12 °C/min to 280 °C which washeld for 1 min. Next, the temperature was increased by 20 °C/min to a final temperature of 300 °C, which was held for 2 min.

Hydrogen stable isotopes of *n*-hexadecane were analyzed by a gas chromatography pyrolysis isotope ratio mass spectrometry (GC-P-IRMS, GC, 7890A series, Agilent Technologies, Santa Clara, CA, USA; IRMS, Thermo Finnigan, Bremen, Germany). The organically bound hydrogen was converted to H_2 by a high temperature pyrolysis at 1420 °C in non-porous alumina tube reactors. 80 mL bacterial culture was sacrificed by the addition of H_2SO_4 (6 M) to a pH around 2 and extracted with 2 mL *n*-hexane by shaking gently at 4 °C for 48 hours. Three to five microliter samples were injected to an injector held isothermally at 250 °C. Standards of *n*-hexadecane with a concentration series of 2 mM, 1.5 mM, 1 mM, 0.5 mM, 0.3 mM, 0.1 mM, 0.05 mM, 0.03 mM and 0.01 mM were prepared in *n*-hexane. A laboratory standard of BTEX mix (for hydrogen isotope analysis: 18 µL of each compound in 10 mL *n*-pentane), which was calibrated

using the international standard and used as quality control of the analytical data, was analyzed before measurement and in between the measurement to ensure data quality of δ^2H values. The GC column and oven temperature program was the same as described for carbon isotope analysis.

2.3.3.7 Isotope calculations

Carbon stable isotope ratios were reported relative to Vienna-Pee Dee-Belemnite (V-PDB) standards as $\delta^{13}C$ values in parts per thousand (‰). Vienna Standard Mean Ocean Water (V-SMOW) was used as the standard for the detection of hydrogen isotope ratios.

$\delta^{13}C$ is defined as:

$$\delta^{13}C = \frac{[R(^{13}C/^{12}C)_{sample} - R(^{13}C/^{12}C)_{std}]}{R(^{13}C/^{12}C)_{std}} \qquad (1)$$

where R_{sample} and R_{std} are the ratios of the heavy isotope to the light isotope ($^{13}C/^{12}C$). δ^2H is defined in the same way as the carbon stable isotope.

$$\delta^{2}H = \frac{[R(^{2}H/^{1}H)_{sample} - R(^{2}H/^{1}H)_{std}]}{R(^{2}H/^{1}H)_{std}} \qquad (2)$$

Bulk carbon enrichment factors (ε_c) were plotted according to the simplified Rayleigh equation:

$$\varepsilon_{bulk} = \frac{\ln(R_t/R_0)}{\ln(C_t/C_0)} \qquad (3)$$

In this equation, R refers to the isotope ratio as described in equation 1 while C refers to the concentration of phenols. The subscripts 0 or t expresses the beginning time point or a certain time point during biodegradation.

Apparent kinetic isotope effect (AKIE) values were calculated as described by equation 4:

$$AKIE = \frac{1}{1 + n \times \varepsilon_{bulk}\,(‰)\,/1{,}000} \qquad (4)$$

Here, n refers to the number of carbon atoms of the analyzed compound.

2.3.3.8 Calculations of mineralized ethylbenzene by the amount of headspace $^{13}CO_2$

The amount of CO_2 produced by mineralization is calculated by the following equation:

$$N_{13CO2} = \left[\left(\frac{R_s}{1+R_s}\right) - \left(\frac{R_0}{1+R_0}\right)\right] \times X_{CO2-Total} \quad (5)$$

$N^{13}CO_2$ is the amount of CO_2 produced by mineralization. Rs is the ratio of heavy isotope to light isotope ($C/^{12}C$) the sampling time point. R_0 is the ratio of heavy isotope to light isotope at the beginning of the experiment. $X_{CO2\text{-}Total}$ is the total amount of CO_2 at the sampling time point.

The amount of mineralized ethylbenzene ($N_{ethylbenzene}$) is calculated by the following equation:

$$N_{ethylbenzene} = N_{13}CO_2/6 \quad (6)$$

(Bombach et al. 2010)

2.4 Molecular biology methods

2.4.1 Isolation of DNA from environmental samples

The extraction was performed with a method described by Maher and colleagues (Maher et al. 2001). Twelve milliliters of culture (liquid and sand mix) was aliquoted into sterile Falcon tubes followed by the addition of 6 mL lysis solution (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% sodium dodecyl sulfate and 0.4 mg/mL proteinase K) into each sample. The mixture was incubated at 55 °C for 2.5 hours. Afterwards, 6 mL Tris-saturated phenol solution (prepared as described by Maniatis et al. (Maniatis et al. 1982)) was added into each mixture and the samples were centrifuged at 3,000 rpm for 10 min at room temperature (Centrifuge 5403, Eppendorf, Hamburg, Germany). The supernatant was collected into another Falcon tube and 3 mL pure chloroform was added into the mixture and after that, another centrifugation (Centrifuge 5403, Eppendorf, Hamburg, Germany) at 3,000 rpm for 10 min at room temperature was conducted. The supernatant was transferred to another Falcon tube and then 300 µL sodium acetate (3 M, pH 5.2) solution, 6 µL of 20 µg/µL glycogen and 7.5 mL ethanol absolute was added into the mixture. Subsequently, the samples were centrifuged at 10,000 x g at 4 °C for 30 min

and supernatant was discarded. 2.5 mL 70 % ethanol was added and a centrifugation at 10,000 x g at 4 °C for 15 min was done after that. The supernatant was discarded and the pellet was dried in a vacuum dryer. 50 µL TE buffer (1 mM EDTA-Na_2, 10 mM Tris-HCl, pH 8.0) was added to dissolve the isolated DNA. The quality and quantity of the yield was checked photometrically using a NanoDrop ND-1000 UV/ Vis spectral photometer (PeqLab, Erlangen, Germany) as well as by standard agarose gel electrophoresis and ethidium bromide staining using UV-light for visualization (E.A.S.Y. RH-3, 254 nm, Herolab, Wiesloch, Germany) (Maniatis *et al.* 1982).

2.4.2 Illumina sequencing

16S rRNA gene fragments were analyzed by Illumina sequencing in cooperation with the working group of Professor Dietmar Pieper, Helmholtz Center for Infection Research, Braunschweig, Germany. The V1 to V2 region of the 16S rRNA gene fragments were amplified using the 27F primers (ACACTCTTTCCCTACACGACGCTCTTCCGATC TCCTGAACAAGAGTTTGATCMTGGCTCAG) with a barcode and 338R primers (GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGCTGCCTCCCGTAGGAGT) (Camarinha-Silva et al. 2014). The amplication was conducted by three polymerase chain reactions (PCR). The first amplification was performed in a total volume of 20 µL containing 4 µL 5x PrimeSTAR TM buffer (Clontech Laboratories, Mountain View, CA, USA), 1.6 µL dNTPs each at a concentration of 2.5 mM (Clontech Laboratories, Mountain View, CA, USA), 0.5 µL each primer at a concentration of 0.2 µM, 1 µL of template DNA (approximately 10 ng) and 0.2 µL PrimeStar HS DNA Polymerase (2.5 U/ µL, Clontech Laboratories, Mountain View, CA, USA) and 12.2 µL MilliQ® water. The PCR program included an initialization step at 95 °C for 3 min which was followed by 20 cycles of denaturation at 98 °C for 10 s and annealing at 55 °C for 10 s and finally extension at 72 °C for 45 s. A second PCR was performed using 1 µL of the mixture from the first PCR and 0.5 µL forward primers with barcode under the same conditions as described above, except that 15 cycles denaturation was used instead of 20. A third PCR aiming at the integration of the Illumina multiplexing sequencing primers and index primers was done in a total volume of 50 µL containing 10 µL 5x PrimeSTAR TM buffer (Clontech Laboratories, Mountain View, CA, USA), 4 µL dNTPs each at a concentration of 2.5 mM (Clontech Laboratories, Mountain View, CA, USA), 1.25 µL each primer at a concentration of 0.2 µM, 1 µL of reaction mixture from last PCR and 0.5µL PrimeStar

HS DNA Polymerase (2.5 U/ µL, Clontech Laboratories, Mountain View, CA, USA) and 32 µL MilliQ® water. The PCR program was the same as described above but 10 cycles of denaturation were used. Negative controls using MilliQ® water as template were performed under the same conditions and were free of products in all rounds of PCR reactions. The PCR products were checked by agarose gel electrophoresis and the correct size (400-500 bp) were cut and recovered using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). The extracts were checked again with agarose gel electrophoresis. The prepared libraries have been undergone sequencing on a GAIIx Genome Analyzer (Illumina, San Diego, CA, USA). For the processing of dataset obtained from the Illumina pipeline, all reads were trimmed to remove the sequences that contain N character, more than 10 homopolymers, mismatches within the primers and barcodes or sequencing errors. Subsequently, all reads were trimmed conservatively to 60nt and matched to give 120nt for downstream analysis. The dataset was obtained as one fasta file.

2.4.3 Sequencing of the 16S rRNA genes

The 16S rDNA sequencing of the ethylbenzene-degrading enrichment cultures from Òdena was carried out by the working group of Dr. Sabine Kleinsteuber, Department of Environmental Microbiology, Helmholtz Center for Environmental Research, Leipzig, Germany. The genomic DNA war extracted and purified from 8 mL enrichment culture with NucleoSpin® Tissue (Macherey-Nagel GmbH & Co.KG, Düren, Germnay) following the support protocol for bacteria in the manufacture instructions except that buffer T1 was exchanged with a buffer for Gram-positive bacteria (20 mM Tris-HCl buffer, pH 8.0, 2 mM EDTA, 1% Triton, 20 mg/ mL lysosome). The bacterial 16S rRNA gene fragments were amplified with the primers 27F (5'-GAG TTT GAT CMT GGC TCA G-3') and 519R (5'-GWA TTA CCG CGG CKG CTG-3'). The sequencing was prepared with ABI PRISMTM BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Deutschland GmbH, Darmstadt, Germany) following the manufacturer instructions (precipitation was done with EDTA/ethanol protocol). Capillary electrophoresis was performed with the ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems Deutschland GmbH, Darmstadt, Germany) and the data were analyzed with ABI PRISM 3130*xl* Data Collection and Sequence Analysis software (Applied Biosystems Deutschland GmbH, Darmstadt, Germany). The contigs were

exported as fasta files and the phylogenic information was acquired by blast using the RDP sequence match tool (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

2.5 Proteomics methods

2.5.1 Protein extraction

For metaproteomic analysis, 1 L Zeitz enrichment culture cultivated with ethylbenzene as substrate under sulfate-reducing condition was used for protein extraction as described (Starke *et al.* 2016) . The enrichment culture was incubated in ultrasonic bath for 10 min with stirring using a spatula to bring attached microorganisms into the water phase. The supernatant was collected into a beaker and the equal volume of ddH_2O (double distilled water) was added and the mixture was incubated again in ultrasonic bath for 10 min. Afterwards, the ultrasonication step was repeated once more. The supernatant was collected and centrifuged at 4 °C, 12,000 xg (Sorvall RC 6 plus, Thermo Electron Corporation, Waltham, MA, USA) for 12 min. The supernatant was discarded and the pellet was resuspended in Tris/HCl (20 mM, pH 7.5) buffer and then undergone an ultrasonication process using an ultrasonic probe on ice for 4 x 5 min at 40 W and 50 % duty cycle (UP50H, Hielscher Ultrasonics GmbH, Teltow, Germany). The mixture was centrifuged again at 4 °C and 12,000 xg for 10 min (Sorvall RC 6 plus, Thermo Electron Corporation, Waltham, MA, USA) and the supernatant was discarded and the pellet was resuspended in Tris/HCl (20 mM, pH 7.5) buffer. An equal volume of phenol solution (10 g/ mL, approx.106.4 M) was added to the mixture and the mixed solution was shaken at room temperature, 500 rpm for 1 hour. A centrifugation was performed at 4 °C, 7830 rpm for 10 min (Sorvall RC 6 plus, Thermo Electron Corporation, Waltham, MA, USA). The lower phase was collected and fivefold of the volume ice cold ammonium acetate (100 mM) in methanol was added to it. The mixture was incubated at – 20 °C overnight and centrifuged at the condition described above (4 °C, 12,000 xg) for 10 min. The supernatant was discarded and the pellet was then washed with ammonium acetate (100 mM) by resuspending with ultrasonication, incubating for 15 min at -20 °C and centrifuging in the conditions as described above (4 °C, 7830 rpm) for 10 min. The washing steps were repeated twice with acetone (80%) and only once with ethanol (70%). The extracted protein pellet was air-dried.

2.5.2 Metaproteomic and protein stable isotope probing (Protein-SIP) analysis

Protein-SIP analysis was performed by the Department of Molecular Systems Biology, Helmholtz Center for Environmental Research, UFZ, Leipzig using a protocol as described (Starke *et al.* 2016). The extracted proteins were prepared for the LC-MS analysis through a SDS gel electrophoresis and proteolytic cleavage of the proteins into peptides using trypsin. The peptide lysates were separated on a UHPLC system (Ultimate 3000, Dionex/Thermo Fisher Scientific, Idstein, Germany). Five µL samples were first loaded for 5 min on the pre-column (µ-pre-column, Acclaim PepMap, 75 µm inner diameter, 2 cm, C18, Thermo Scientific) at 4% mobile phase B (80% acetonitrile in Nanopure water with 0.08% formic acid), 96% mobile phase A (Nanopure water with 0.1% formic acid), then eluted from the analytical column (PepMap Acclaim C18 LC Column, 25 cm, 3 µm particle size, Thermo Scientific) over a 150 min non-linear gradient of mobile phase B (4-55% B). Mass spectrometry (MS) was performed on an Orbitrap Fusion MS (Thermo Fisher Scientific, Waltham, MA, USA) with a TriVersa NanoMate (Advion, Ltd., Harlow, UK) source in LC chip coupling mode. The MS was set at cycle time of 3 s used for MS/MS scans with higher energy collision dissociation (HCD) at normalized collision energy of 28%. MS scans were measured at a resolution of 120,000 in the scan range of 350-2,000 m/z. MS ion count target was set to 4×105 at an injection time of 100 ms. Ions for MS/MS scans were isolated in the quadrupole with an isolation window of 1.6 Da and were measured with a resolution of 15,000 in the scan range of 350-1,400 m/z. The dynamic exclusion duration was set to 30 s with a 10 ppm tolerance. Automatic gain control target was set to 6×104 with an injection time of 150 ms using the underfill ratio of 1%.

Peptide identification was performed using Thermo Proteome Discoverer (v1.4.0.288; Thermo Fisher Scientific, Waltham, MA, USA). Database searches were performed by using the Sequest HT algorithm with the following parameters: tryptic cleavage with maximal two missed cleavages, a peptide tolerance threshold of ±10 ppm and an MS/MS tolerance threshold of ±0.1 Da, and carbamidomethylation at cysteines as static and oxidation of methionines as variable modifications. Searches were performed against bacterial and archaeal sequences of the NCBI non-redundant database (v 22, April 2014, National Library of Medicine, Bethesda, MD, USA) and nucleic acid shotgun metagenome database from the benzene-degrading and *m*-xylene-degrading communities

enriched from the same contaminated aquifer (Taubert *et al.* 2012, Bozinovski et al. 2014). The MetaProSIP node from OpenMS was used to detect the ^{13}C patterns in the MS spectra and to calculate the relative isotope abundance and labelling ratio for each detected peptide (Sachsenberg et al. 2015).

2.6 Field sampling methods

2.6.1 Sampling for enrichment cultures in Zeitz, Germany

The investigated contaminated aquifer is located at a former coal hydrogenation and benzene production plant near Zeitz, Saxony-Anhalt, Germany. There are two aquifers (lower and upper) present at the contaminated site which were separated but still partially connected by a discontinuous lignite-clay layer. The aquifers contain river gravel and sand sediments (more than 95% quartz in the composition) and the groundwater flows to the northeast direction. Due to an air raid in the Second World War as well as several damages and accidents during the operation of the plant between 1960 and 1990, the two aquifers were heavily contaminated with BTEX compounds (benzene and toluene concentrations up to 950 and 50 mg/ L, respectively, approx. 12 mM and 0.5 mM). The aquifers are recognized as anoxic and have sulfate concentration as high as 10 mM, probably due to previous lignite mining. Sulfate was therefore identified as the main electron acceptor of the site (Schirmer et al. 2006). The monitoring of natural attenuation (NA) processes at this site has been conducted intensively during the recent years and in 2002, an experimental plant was built by the UFZ in order to investigate the bioremediation in the lower aquifer. A newer column system made of four stainless steel columns (190 cm x 27.3 cm) was started in 2006 for the further study of NA processes in Zeitz. The sand material for the enrichment of the ethylbenzene-degrading culture was taken from this system. The columns were filled with sand granules with the size in average between 2 and 3.15 mm. Groundwater from the lower aquifer has been pumped into the columns at a flow rate of 0.5 L/h (Taubert *et al.* 2012). The groundwater contained BTEX contaminants with an average of 300 µM benzene and trace amount ($\leqslant$ 1 µM) of toluene, ethylbenzene and xylenes (Vogt *et al.* 2007). Moreover, there were 4 mM sulfate, 300 µM sulfide and 120 µM ammonium present (Vogt *et al.* 2007). The BTEX compounds were reported to be degraded under sulfate-reducing conditions (Vogt *et al.* 2007, Herrmann et al. 2009, Bombach *et al.* 2010, Dorer *et al.* 2014). For the

enrichment of the ethylbenzene-degrading, sulfate-reducing community, the sand granules were transferred into sterile 1 L Schott bottles, filled with anoxic groundwater and closed with butyl stoppers and screw caps. Sand materials for setting up of the enrichment cultures were taken from the top layer of the column using a sterile spoon from an opened sample port. The liquid phase was then replaced in the anoxic glove box with anoxic mineral salt medium as described in chapter 2.2.1.

2.6.2 Multilevel-sampling at field site in Òdena, Spain

The investigated contaminated aquifer is located at a former chemical plant in the town Òdena which is 50 km northwest of Barcelona, Spain. The site is heavily contaminated due to large scale consuming of tetrachloroethylene (PCE) and trichloroethylene (TCE) as intermediates for the manufacture of phytosanitary products and chemicals for the textile industry between the year of 1978 and 1985. The site is highly complex with multiple contaminant sources and multiple sorts of contaminants which include chlorinated methanes, chlorinated ethane, chlorinated benzenes, chlorinated ethanes, BTEX compounds and pesticides. The aquifer is formed with an Ecocene blue-grey limestone bed with low permeability and groundwater at depths between 3.2 m and 11.7 m below ground surface. The flow rate of the groundwater ranges from $1.44x10^{-3}$ to 0.6 m/day (Palau et al. 2014). The schematic picture of the site is shown in Figure 2.1.

Bioremediation of contaminants at this site has been extensively studied by the working group of Prof. Dr. Albert Soler, Department of Mineralogy, Petrology and Applied Geology, University Barcelona. The sampling at the field site and subsequent analysis was performed in cooperation with this working group. The groundwater samples for isotope analysis were taken from 3 multilevel nested wells (S3, S6, and S9, Figure 2.1) through manually purging with syringes from multilevel tube samplers or through a bailer (Figure 2.2). Moreover, sludge and groundwater samples for setting up microcosms experiments were taken through pumping from the bottom of each well. Eh, pH, dissolved oxygen and temperature were measured in situ before sampling. The equipment and methods used with the multilevel-sampler was developed and described by the group of Prof. Dr. Albert Soler (Palau *et al.* 2014). The schematic picture of the sampling site is shown in Figure 2.2.

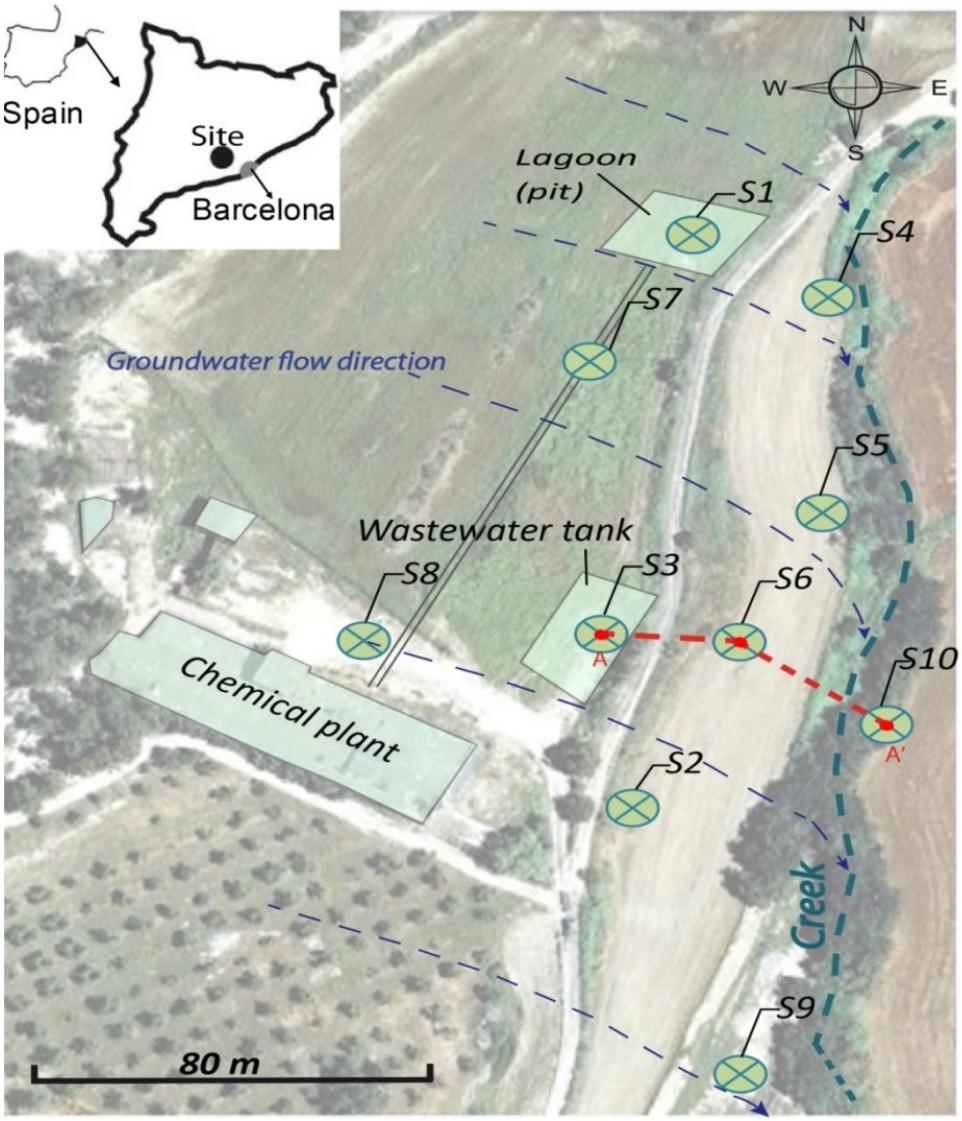


Figure 2.1. The schematic picture of the Òdena contaminated site according to Rodríguez-Fernández and colleagues (Rodríguez-Fernández et al. 2015). Multilevel sampling wells are marked with ⊗. Sample in this study were taken from wells S3, S6 and S9.

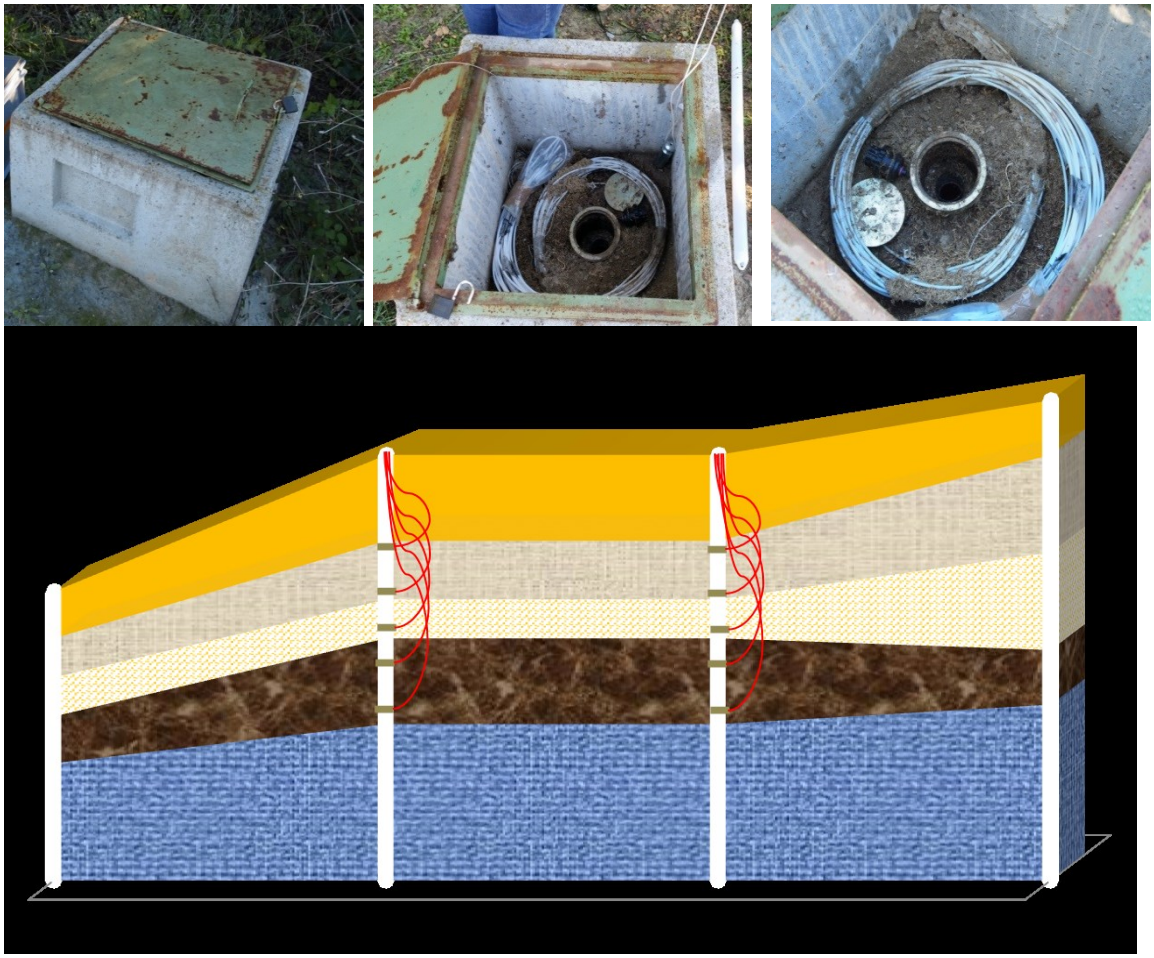


Figure 2.2. The schematic picture of the Òdena multilevel sampling wells (lower) and the photos of the sampling well and multilevel sampling tubes (upper).

3 RESULTS

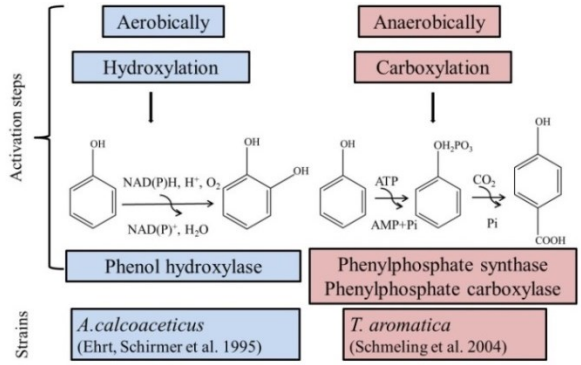
3.1 Identification of biodegradation pathways by compound specific isotope analysis

3.1.1 Identification of biodegradation pathways of phenol and cresols by compound specific stable carbon isotope analysis using LC-IRMS

The aim of this study was to provide a first data set for carbon isotope fractionation upon the initial steps of different aerobic and anaerobic activation steps of phenol and cresol degradation pathways. For this purpose, a LC-IRMS-based method for carbon isotope analysis of phenol and cresols was firstly developed. Aerobic hydroxylation of the aromatic ring of phenol and *p*-cresol and aerobic hydroxylation of the methyl moiety of *p*-

cresol was investigated using *Acinetobacter calcoaceticus* NCIMB8250 and *Pseudomonas sp.* NCIMB 9867, respectively. The anaerobic carboxylation of phenol was examined using *Thauera aromatica* DSM 6984 and *Desulfosarcina cetonica* DSM 7267. Anaerobic activation of the methyl moiety of cresols by fumarate addition was investigated in experiments with *D. cetonica,* and O_2-independent hydroxylation was tested with *Azoarcus buckelii* DSM 14744 and *Geobacter metallireducens* DSM 7210, respectively. The investigated initial steps of the different degradation pathways are shown in figure 3.1. The obtained fractionation factors allow an evaluation of the potential of stable isotope fractionation analysis for monitoring of phenols biodegradation in the environment.

Overview of phenol degradation activation mechanisms



Overview of *p*-cresol degradation activation mechanisms

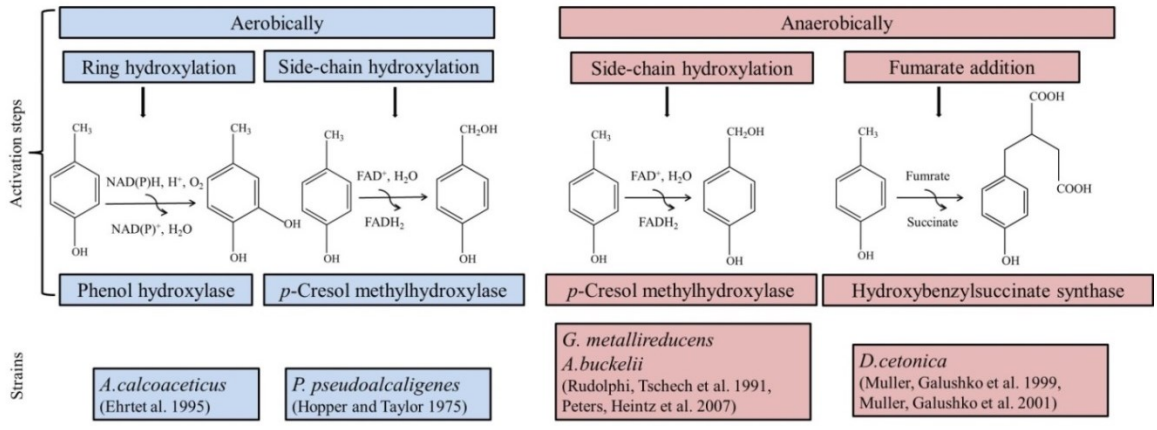
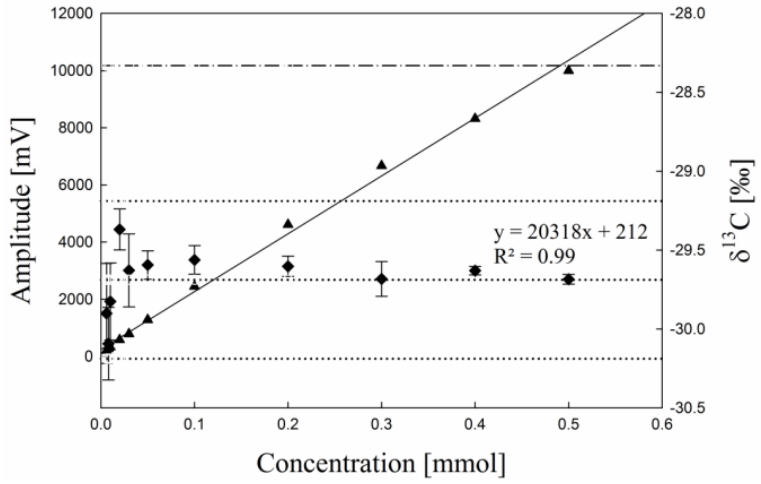


Figure 3.1 Initial steps of microbial phenol and *p*-cresol degradation pathways investigated in this study and the strains selected for the study of isotope fractionation upon each activation mechanism as well as the enzymes involved. Reactions in blue color are aerobic reactions and the ones in red are anaerobic reactions (Wei et al. 2016).

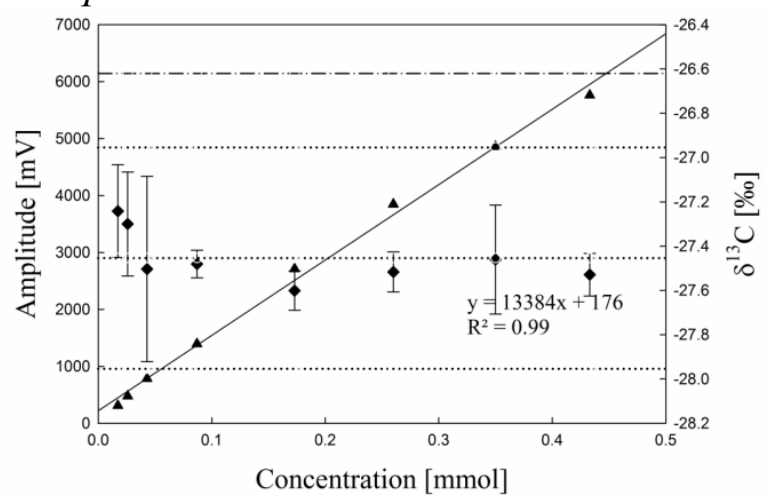
3.1.1.1 Method validation of phenol and cresols carbon isotope measurement by LC-IRMS

All the $\delta^{13}C$ values were measured with an accuracy of ⩽1.4 ‰ and precision of ⩽± 0.4 ‰. The Method Detection Limits (MDL), based on moving mean procedure as described elsewhere(Jochmann et al. 2006), were 142 ng and 43 ng of carbon on column, which corresponds to peak amplitudes of m/z 44 of ~350 mV and ~200 mV for cresols and phenol, respectively (Figures 5.2). MDLs of the phenols and cresols are given in Table 3.1. Comparing the results obtained by EA and LC-IRMS for standards, the highest offset in $\delta^{13}C$-values was observed for phenol (-1.4‰); for *m*-cresol, the isotopic shift was 1‰, and for *p*-cresol and *o*-cresol less than 1 ‰. In order to check if there is any consistent trend in changes of isotope values with the change of concentration, a linear regression model was applied. In case of *m*-cresol (y=1.0657x – 28.3) and phenol (y= 0.2026x – 29.7), a linear increase of isotope values with the concentration increase was observed. The opposite trend, slight decrease in isotope values with the increase of concentration, was observed for *o*-cresol and for *p*-cresol, with regression equation y= -1.0026x-28.0 and y= -0.415x -27.4, respectively. Nevertheless, the standard deviation of $\delta^{13}C$ values between the lowest and the highest measured concentration was less than 0.4‰ for all measured compounds, therefore no correction was applied. It is concluded that phenol and cresols were quantitatively converted into CO_2 in the LC-IRMS interface.

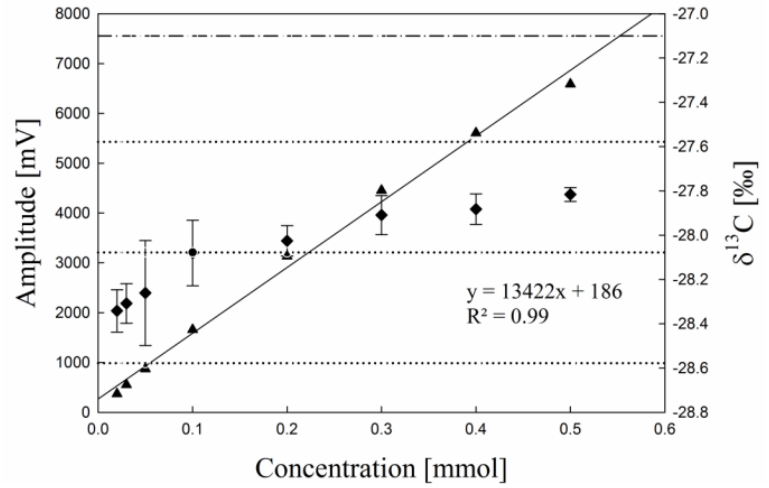
a phenol



b *p*-cresol



c *m*-cresol



d *p*-cresol

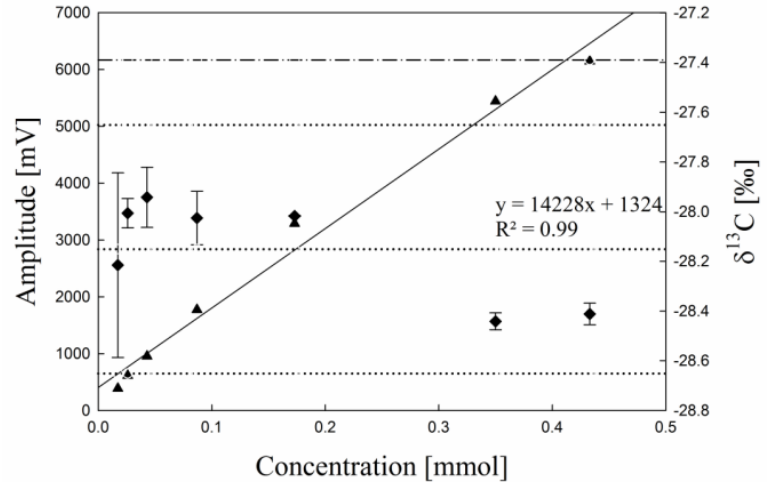


Figure 3.2. Representative calibration line of (a) phenol, (b) *p*-cresol, (c) *m*-cresol and (d) *o*-cresol; triangles correspond to peak amplitudes of the *m/z* 44 trace whereas diamonds relate to $\delta^{13}C$ values. The dashed lines represent the interval of $\delta^{13}C$ mean value ± 0.5‰. The dashed dotted line shows the $\delta^{13}C$ EA-IRMS value (Wei *et al.* 2016).

Table 3.1 Method parameters for carbon stable isotope analysis of phenol and cresols

	MDL* (ng of C on column)	MDL* (nmol)	Minimum peak amplitude of *m/z* 44 for accurate and precise isotope values [mV]	EA-IRMS value [‰]	LC-IRMS value [‰]	Isotopic shift [‰]
Phenol	43	0.6	210	-28.3 ± 0.1	-29.8 ± 0.2	1.4
m-Cresol	143	1.7	370	-27.1 ± 0.1	-28.1 ± 0.2	1.0
p-Cresol	143	1.7	310	-26.6 ± 0.1	-27.5 ± 0.1	0.8
o-Cresol	143	1.7	380	-27.4 ± 0.1	-28.2 ± 0.2	0.8

* MDL: method detection limit

3.1.1.2 Carbon isotope fractionation upon aerobic biodegradation of phenol and cresols

A. calcoacticus and *P. pseudoalcaligenes* degraded under the given experimental conditions phenol or *p*-cresol within 10 to 20 hours (Figure 3.3 a, b, c). The degradation of more than 99% of 1 mM phenol by *A. calcoacticus* was accompanied by a change in the $\delta^{13}C$ value from the initial value of -29.5‰ to a value of -21.3‰. Carbon isotope enrichment factors and $AKIE_C$ values were calculated as -1.5 ‰ ± 0.05 ‰ and 1.0091 ± 0.0003 (Table 3.2). Similarly, degradation of 92% of 1 mM *p*-cresol by *A. calcoacticus* was associated by an enrichment of $\delta\ ^{13}C$ from -27.9 ‰ to -24.3 ‰. The resulting enrichment factors and $AKIE_C$ values were -1.4 ‰ ± 0.2 ‰ and 1.0099 ± 0.0014, respectively (Table 3.2). *p*-Cresol degradation by *P. pseudoalcaligenes* resulted in a $\delta\ ^{13}C$ value enrichment from -27.65 ‰ to -24.77 ‰ upon 75% degradation of 1 mM *p*-cresol leading to carbon isotope enrichment factors and $AKIE_C$ values of -2.3 ‰ ± 0.19 ‰ and 1.0164 ± 0.0014 (Table 3.2).

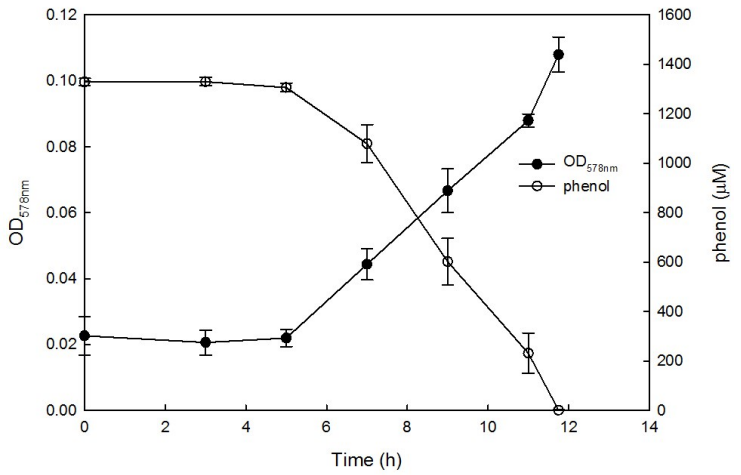
3.1.1.3 Carbon isotope fractionation upon anaerobic biodegradation of phenol and cresols

Under the experimental conditions, anaerobic biodegradation of phenol and cresol is slower compared to the experiments with the aerobic cultures; the substrates were completely degraded within 3 to 20 days (Figure 3.4). *T. aromatica* grown under nitrate-reducing condition degraded 96% of the added phenol within 20 days (Figure 3.4, d), producing an average of enrichment of +2.1 ‰ in the $\delta\ ^{13}C$ value relative to the initial carbon isotope composition of -29.2 ‰. *G.metallireducens* and *A.buckelii* accumulated nitrite in the course of *p*-cresol degradation (Figure 3.4). *G.metallireducens* degraded *p*-cresol (0.5 mM) under nitrate-reducing conditions in 14 days (Figure 3.4, f). Degradation of 80 % of *p*-cresol was accompanied by an increase in $\delta\ ^{13}C$ values of 5.6 ‰. The corresponding enrichment factors and $AKIE_C$ values were -3.6 ‰ ± 0.36 ‰ and 1.0259 ± 0.0027, respectively (Table 3.2). In comparison, at about 93% *p*-cresol degradation in 4 days (Figure 3.4, e), *A.buckelii* yielded an enrichment of 5.2 ‰ and enrichment factors -2.0 ‰ ± 0.14 ‰ and $AKIE_C$ values 1.0142 ± 0.0010 were obtained (Table 3.2). *D.cetonica* cultures were established under sulfate-reducing conditions using phenol, *m*-cresol or *p*-cresol as the sole source of carbon and energy. Substrate concentrations were always 0.5 mM. Complete degradation of phenol took 10 to 15 days (Figure 3.4, a, b, c). The initial carbon isotope composition of was -28.8 ‰. Noticeably, a small but reproducible inverse carbon isotope fractionation of 1.6 ‰ was detected, producing an enrichment factor and $AKIE_C$ value of 0.4 ‰ ± 0.08 ‰ and 0.9976 ± 0.0005, respectively (Table 3.2). The degradation patterns of the two cresols resembled each other. It took 8 days for 70% of substrate depletion (Figure 3.4, a, b). An enrichment of ^{13}C from -27.27 ‰ to -24.39 ‰ for *m*-cresol and from -27.4 ‰ to -24.85 ‰ for *p*-cresol was recorded. The obtained enrichment factors were -2.2 ‰ ± 0.31 ‰ for *m*-cresol degradation and -1.9 ‰ ± 0.15 ‰ for *p*-cresol degradation; furthermore, the calculated $AKIE_C$ values were 1.0156 ± 0.0023 and 1.0135 ± 0.0011, respectively (Table 3.2).

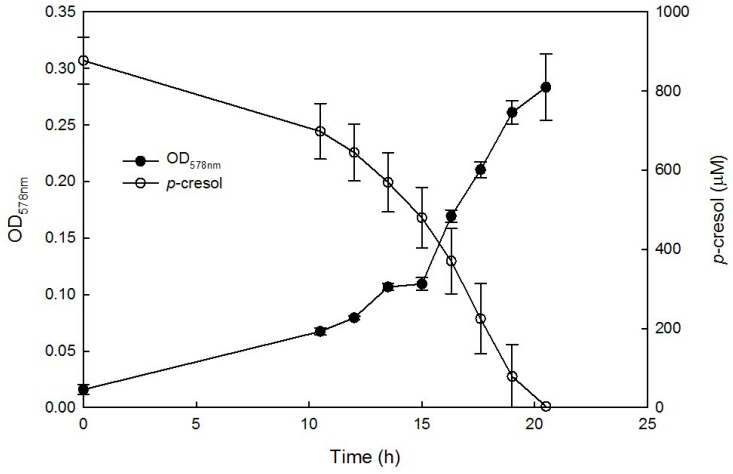
Concentration decrease and changes in $\delta^{13}C$ values in abiotic controls were not observed (data not shown), confirming that no other processes besides biodegradation caused the carbon isotope fractionation in the system.

Rayleigh plots of the degradation pattern are shown in Figure 3.5. All ε_c and $AKIE_C$ values generated in this study are summarized in Table 3.2. The distribution of carbon stable isotope fractionation upon different degradation mechanisms is shown in Figure 3.6.

a



b



c

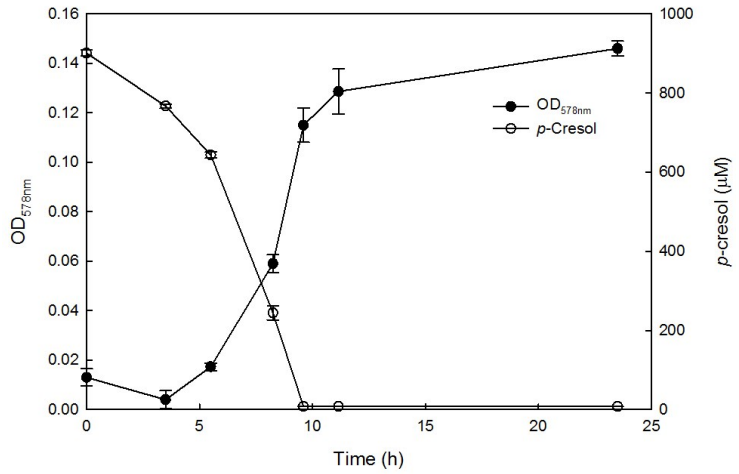
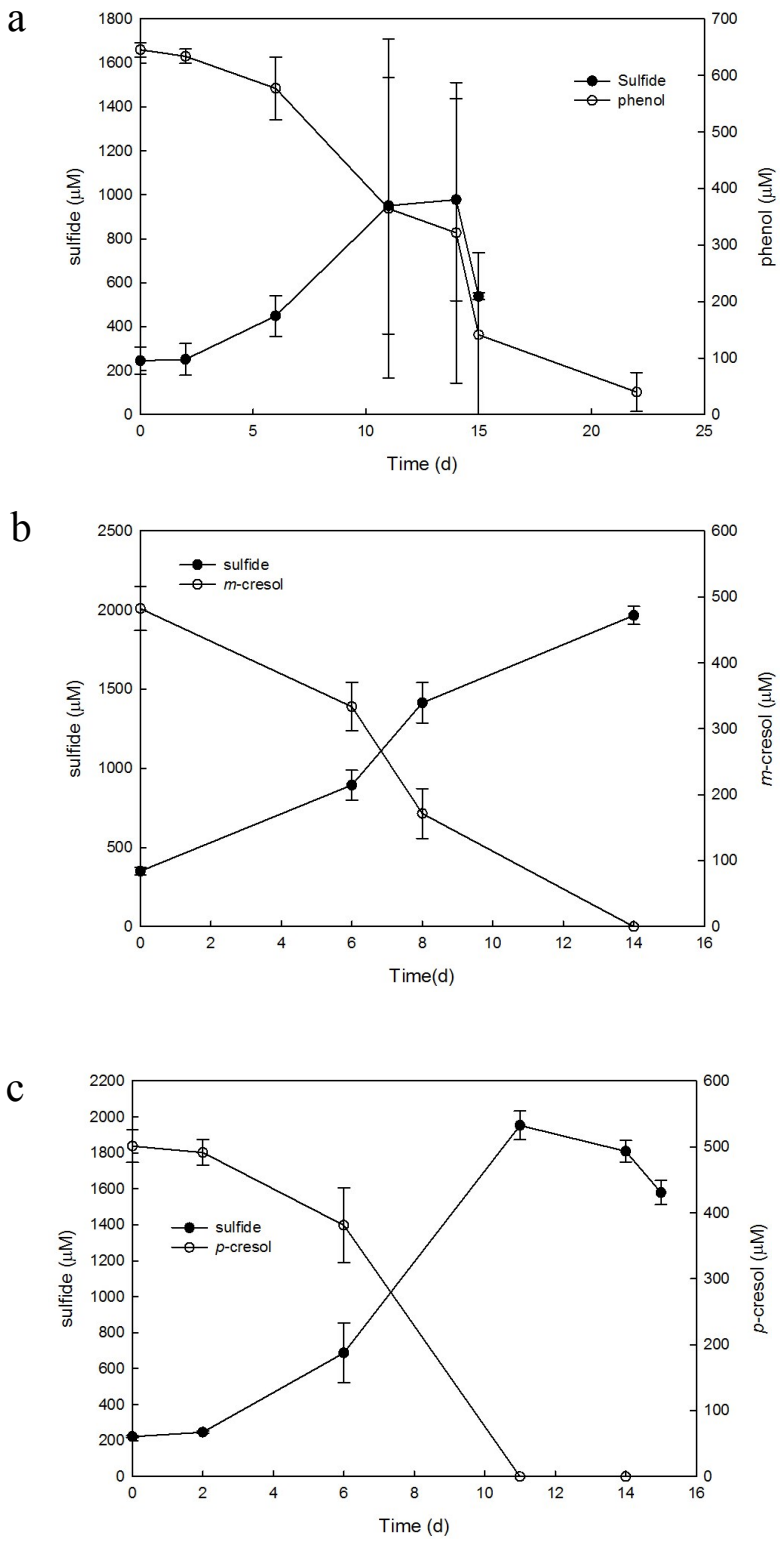


Figure 3.3. Degradation kinetics of phenol and cresols by the studied aerobic strains. The substrate concentration and optical density were measured in triplicates. The average values as well as standard deviations were calculated and plotted in the figure. a) *A. calcoaceticus*, degradation of phenol; b) *A. calcoaceticus*, degradation of *p*-cresol; c) *P.pseudoalcalignes*, degradation of *p*-cresol (Wei *et al.* 2016).



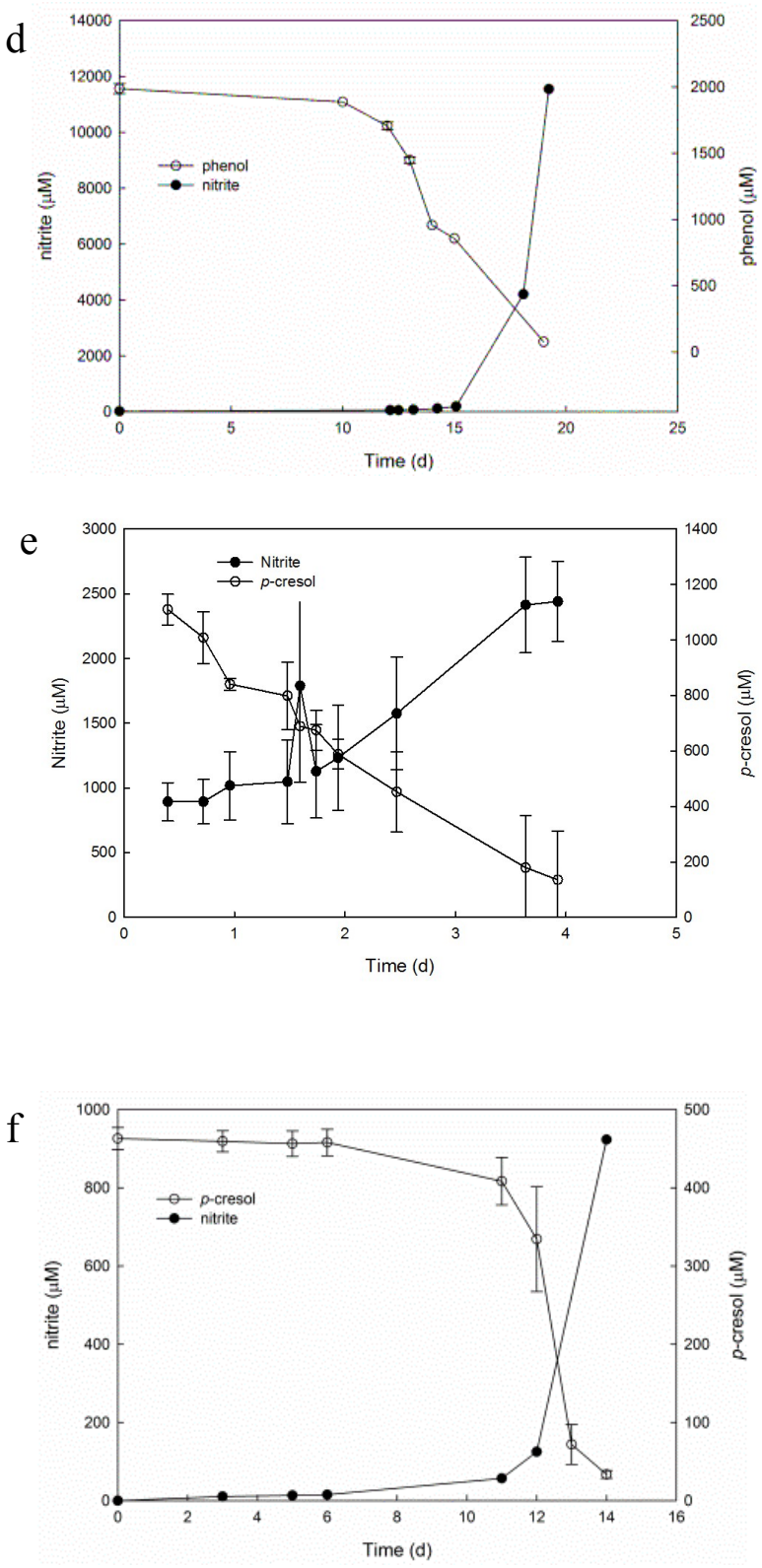
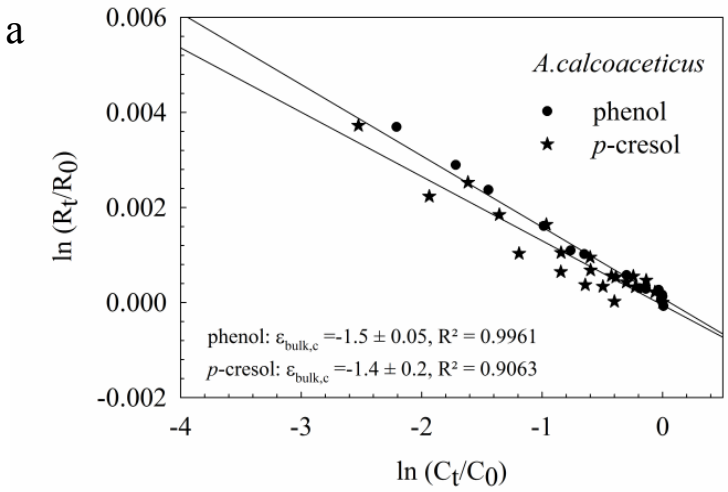
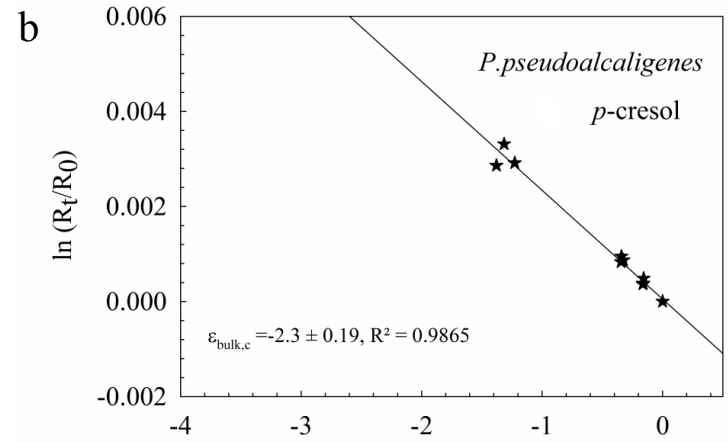
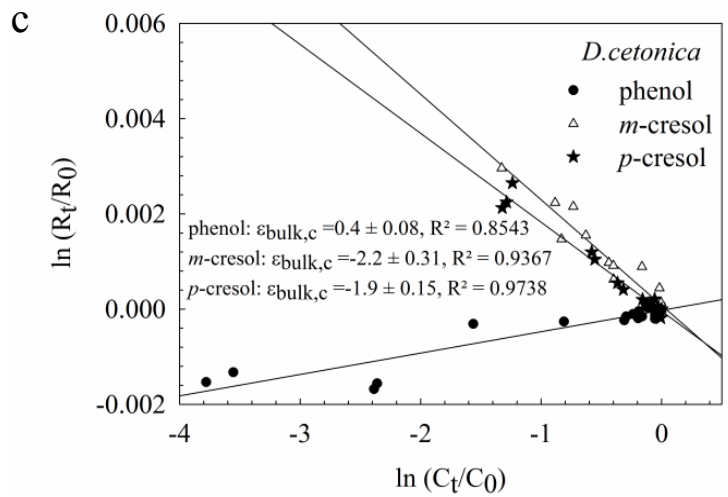


Figure 3.4. Degradation kinetics of phenol and cresols by the studied anaerobic strains (Wei *et al.* 2016). Nitrite and sulfide, which were the reduction products coupled to the phenols degradation process, were used as the indication of culture growth. The substrate and products concentration were measured in triplicates. The average values as well as standard deviations were calculated

and plotted in the figure. a) *D.cetonica*, degradation of phenol; b) *D.cetonica*, degradation of *m*-cresol; c) *D.cetonica*, degradation of *p*-cresol; d) *T.aromatica*, degradation of phenol; e) *A. buckelii*, degradation of *p*-cresol; f) *G.metallireducens*, degradation of *p*-cresol.







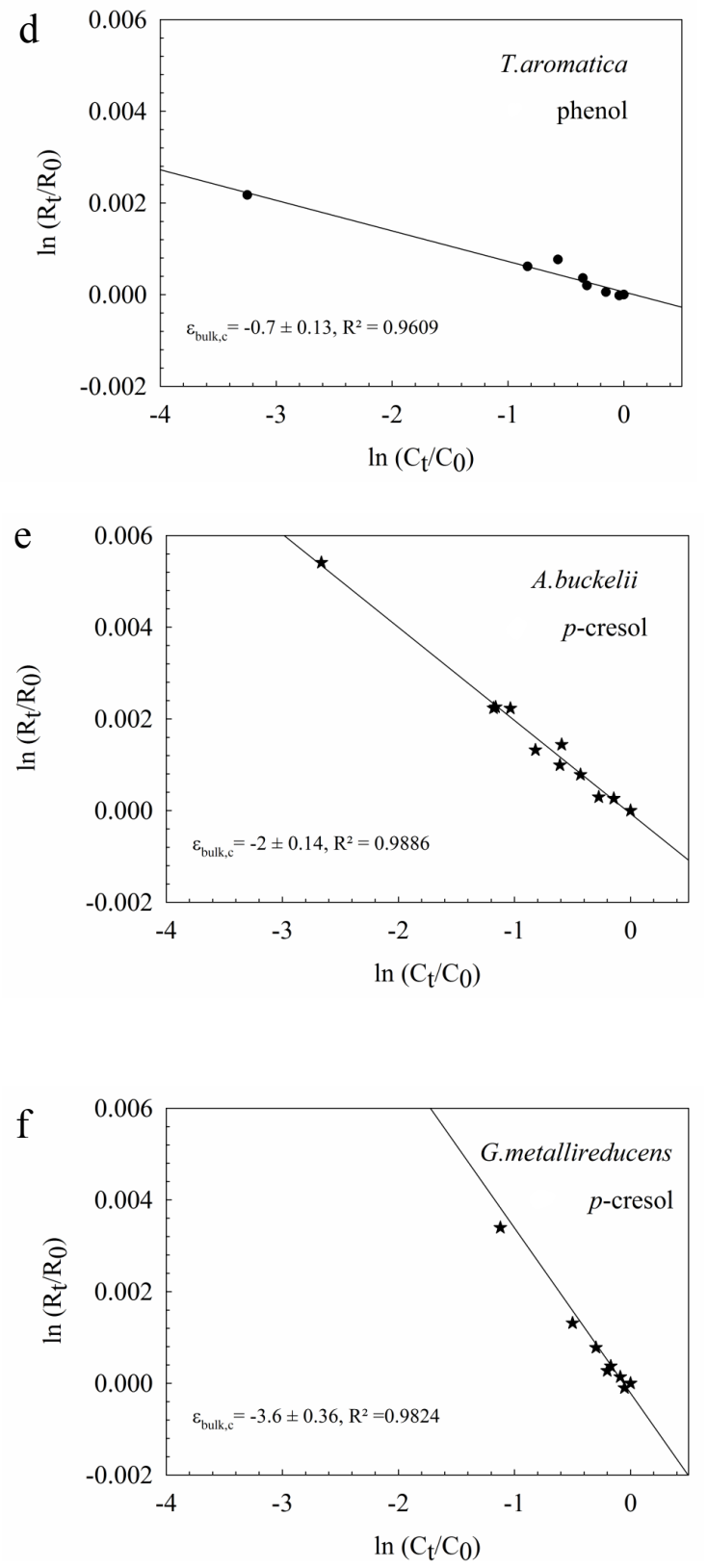


Figure 3.5. Rayleigh plots of carbon isotope fractionation for each strain and substrate examined in this study (Wei *et al.* 2016). Bulk enrichment factors (‰) were calculated from the slope of the regression curve and linearity is expressed in r^2 values. a) *A. calcoaceticus*, aerobic degradation of phenol and *p*-cresol; b) *P.pseudoalcalignes*, aerobic degradation of *p*-cresol; c) *D.cetonica*,

anaerobic degradation of phenol, *m*-cresol and *p*-cresol; d) *T.aromatica*, anaerobic degradation of phenol; e) *A. buckelii*, anaerobic degradation of *p*-cresol; f) *G.metallireducens*, anaerobic degradation of *p*-cresol.

Table 3.2 Bulk enrichment factor and $AKIE_C$ values upon phenol and cresols degradation investigated.

Microorganism	Substrate and terminal electron acceptor	Investigated enzymatic reaction (initial step(s) of the degradation pathway)	$\varepsilon_{bulk,c}$ (‰)	$AKIE_C$
A. calcoaceticus	phenol, oxygen	hydroxylation, reaction with molecular oxygen	-1.5 ± 0.1	1.0091 ± 0.0003
A. calcoaceticus	*p*-cresol, oxygen	ring hydroxylation, reaction with molecular oxygen	-1.4 ± 0.2	1.0099 ± 0.0014
P. pseudoalcaligenes	*p*-cresol, oxygen	side chain hydroxylation with water	-2.3 ± 0.2	1.0164 ± 0.0014
D. cetonica	phenol, sulfate	phosphorylation followed by carboxylation (hypothesized)	0.4 ± 0.1	0.9976 ± 0.0005
D. cetonica	*m*-cresol, sulfate	fumarate addition	-2.2 ± 0.3	1.0156 ± 0.0023
D.cetonica	*p*-cresol, sulfate	fumarate addition	-1.9 ± 0.2	1.0135 ± 0.0011
T.aromatica	phenol, nitrate	phosphorylation followed by carboxylation	-0.7 ± 0.1	1.0042 ± 0.0008
A.buckelii	*p*-cresol, nitrate	side chain hydroxylation with water	-2.0 ± 0.1	1.0142 ± 0.0010
G.metallireduces	*p*-cresol, nitrate	side chain hydroxylation	-3.6 ± 0.4	1.0259 ± 0.0027

		with water		

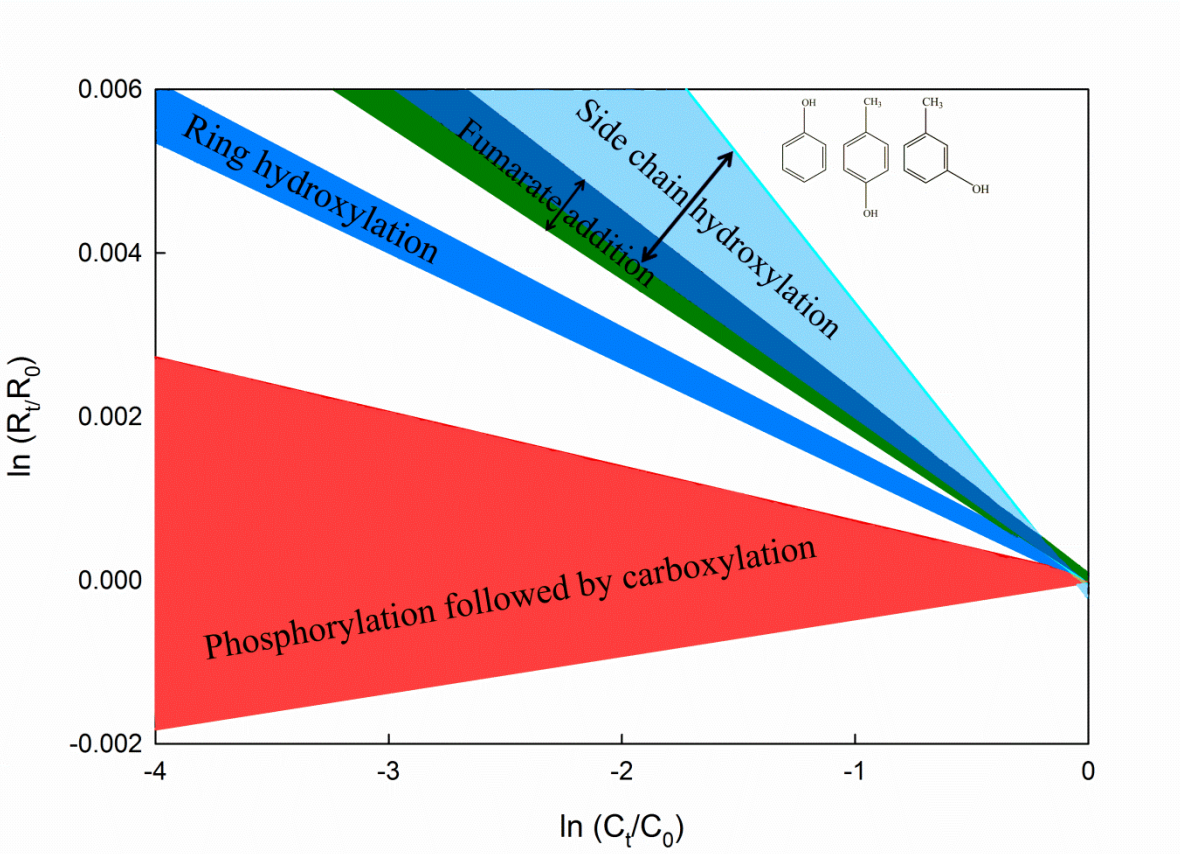


Figure 3.6 Distribution of carbon stable isotope fractionation upon different biodegradation mechanisms of phenol and cresols (Wei *et al.* 2016).

3.1.2 Method development and validation of phenol and cresols hydrogen isotope measurement via GC-IRMS

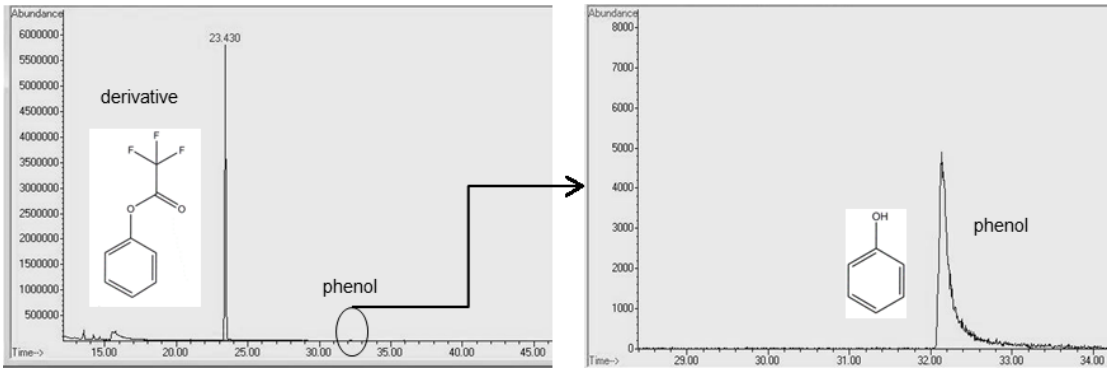
Hydrogen isotope analysis of phenols with the same method developed for carbon isotope analysis using LC-IRMS is not possible. External hydrogen atoms will be introduced through the mobile phase. Thus, the aim of this part of study was to develop a valid method for hydrogen isotope analysis for phenols based on derivatization and subsequent analysis by GC-IRMS. To reduce the polarity and increase the volatility of phenols for GC analysis, derivatization by acetylation was performed with the reagent trifluoroacetic anhydride (TFAA). The mechanism of this reaction is show in chapter 1.3.1.3. The products of the derivatization were analyzed by GC-MS. The hydrogen isotope of the derivatives of different concentration was analyzed and compared to the values determined with the elemental analyzer. To verify the applicability of the method to

analyze the biodegradation process, a measurement of hydrogen isotope fractionation upon biodegradation of *m*-cresol by *D.cetonica* was performed.

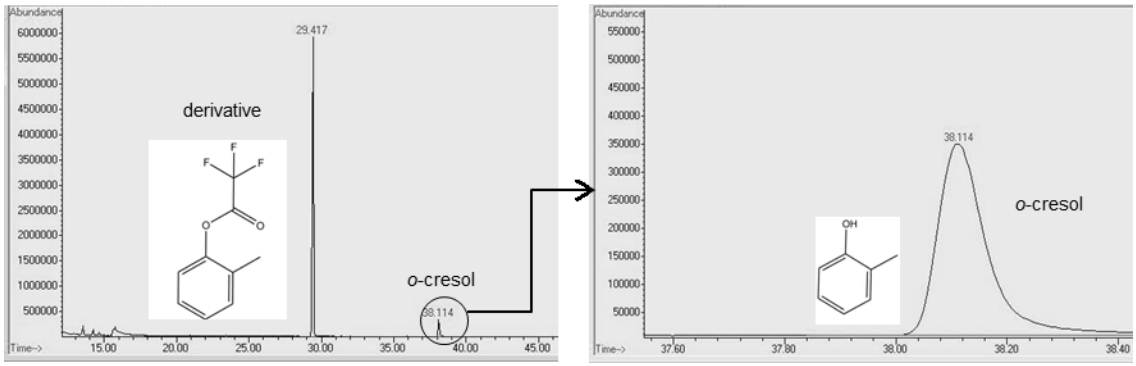
3.1.2.1 Derivatization of phenols using trifluoroacetic anhydride as reagents

The derivatives of phenols were detected by GC-MS (chromatograms shown in Figure 3.7 and mass spectra of derivatives shown in Figure 3.8).The efficiency of derivatization reactions under given condition was above 95% based on calculation from GC-MS measurement. Calibration curves of concentration of derivatives are shown in Figure 3.9 and the results showed that the efficiency of the derivatization reaction is independent of substrate concentration under the given conditions.

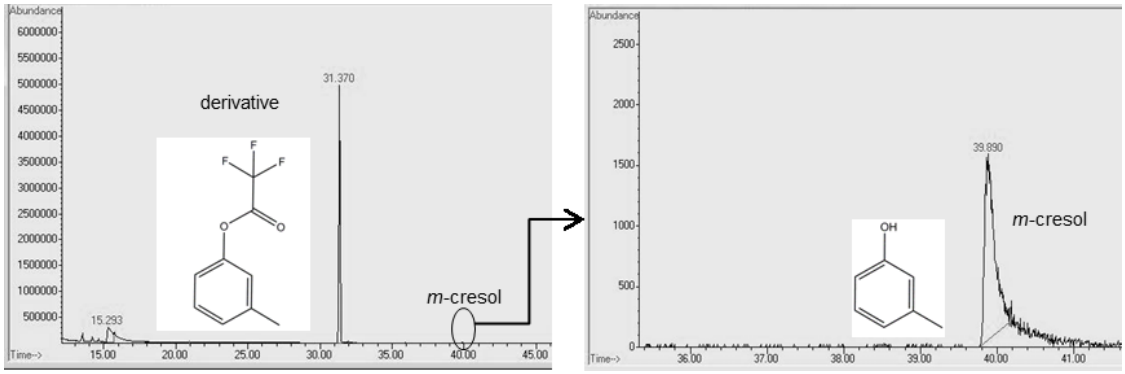
a phenol



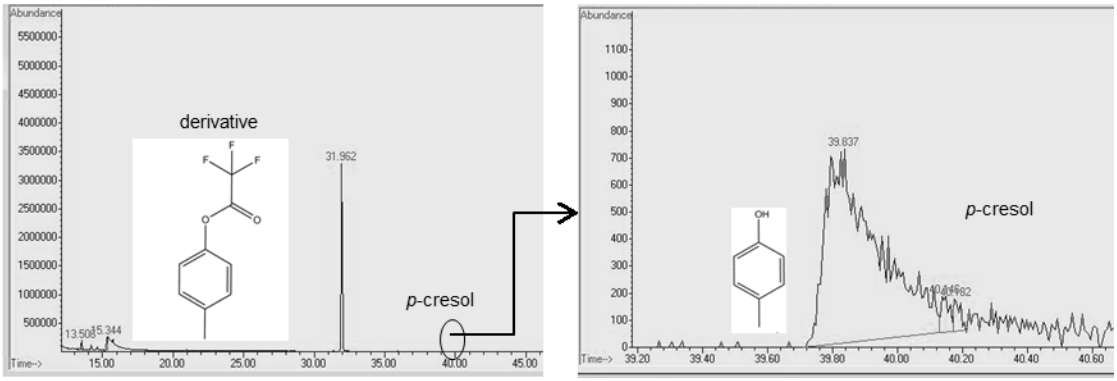
b *o*-cresol



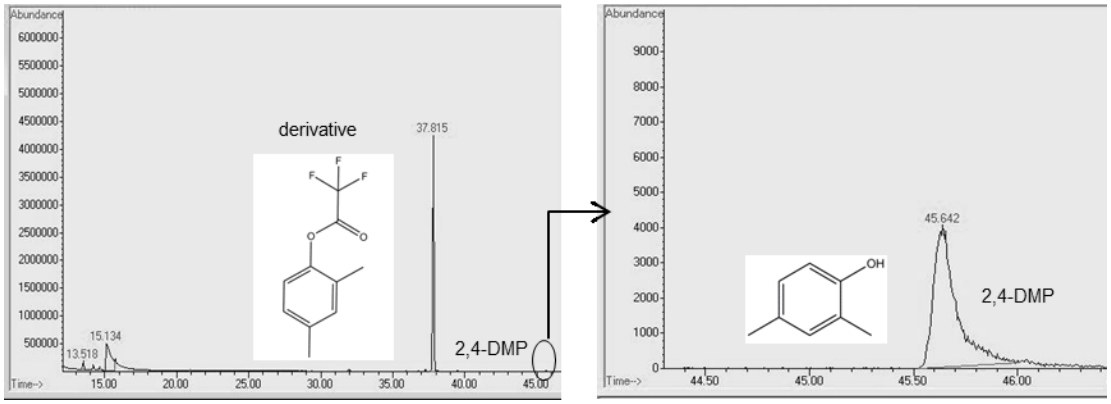
c *m*-cresol



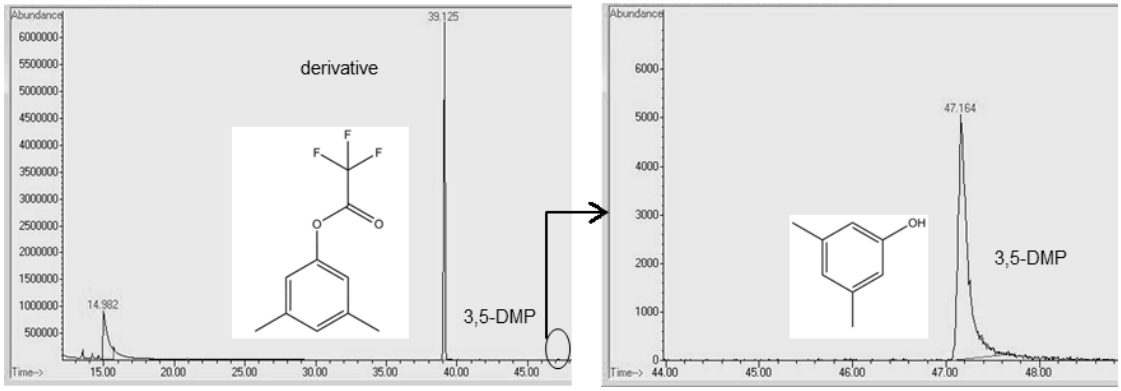
d *p*-cresol



e 2,4-dimethylphenol



g 3,5-dimethylphenol



f 2,5-dimethylphenol

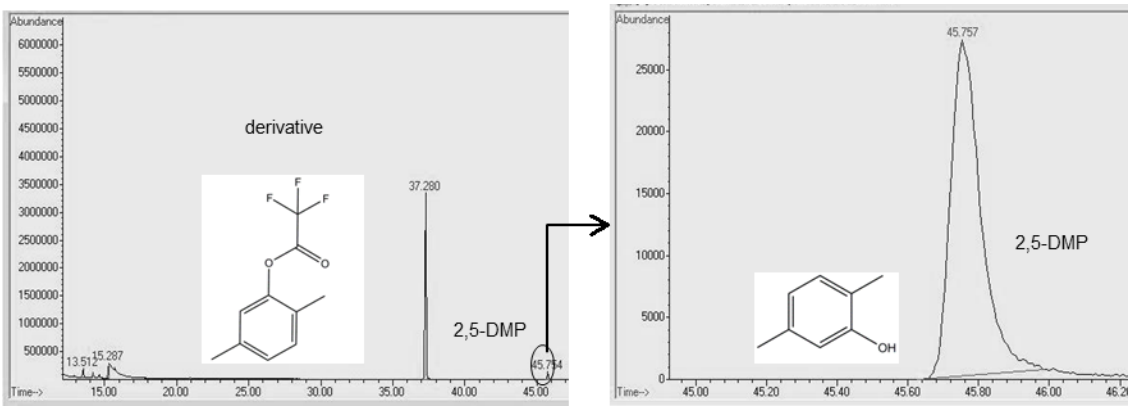
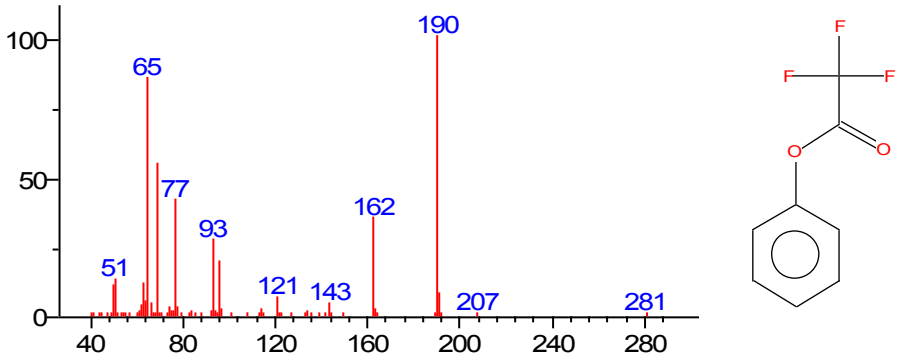
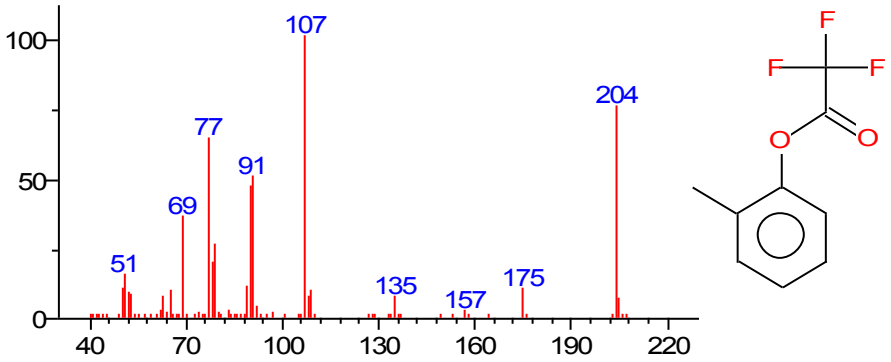


Figure 3.7 Chromatograms of products of the phenols derivatization reaction: a. phenol; b.*o*-cresol; c. *m*-cresol; d. *p*-cresol; e. 2,4-dimethylphenol; f. 2,5-dimethylphenol; g. 3,5-dimethylphenol. The phenols were of a concentration of 1 mg/mL in benzene. The preparation of standards and reaction are described in chapter 2.3.3.5. For each reaction, the picture on the left side shows the chromatogram of the derivative of the corresponding compound and the picture on the right side shows the enlarged chromatogram of the non-reacted substrate.

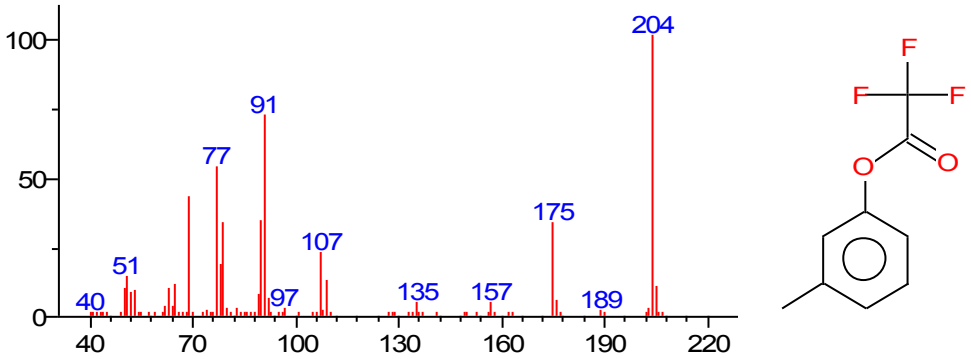
a phenol



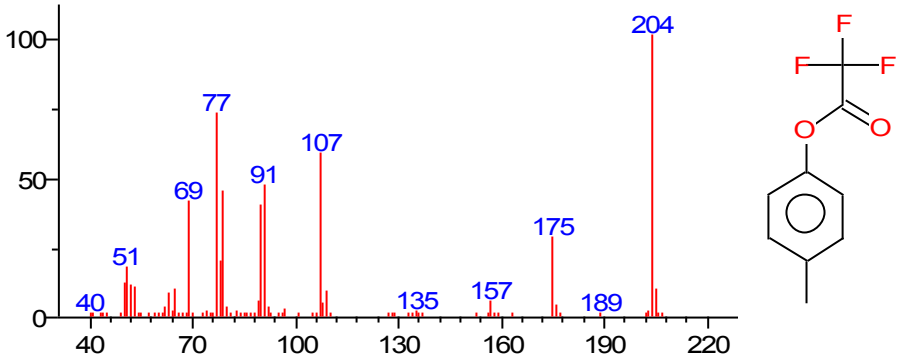
b *o*-cresol



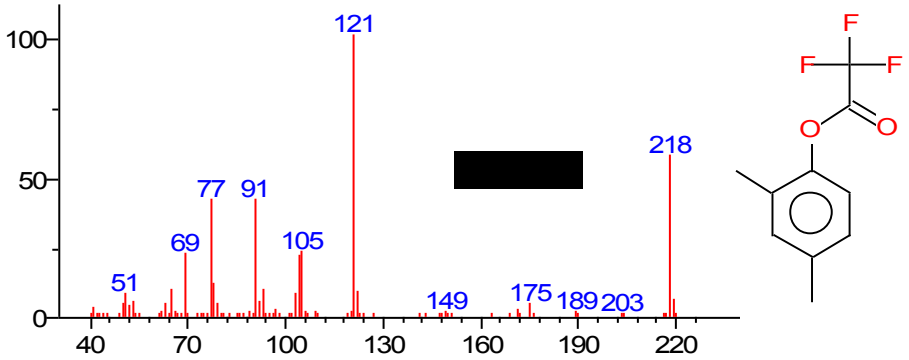
c *m*-cresol



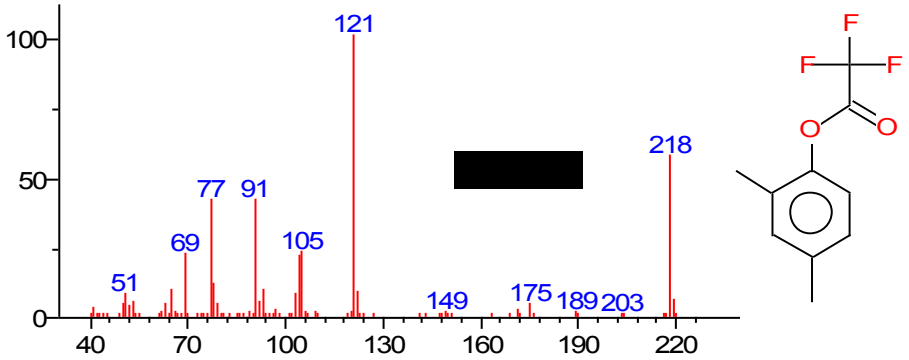
d *p*-cresol



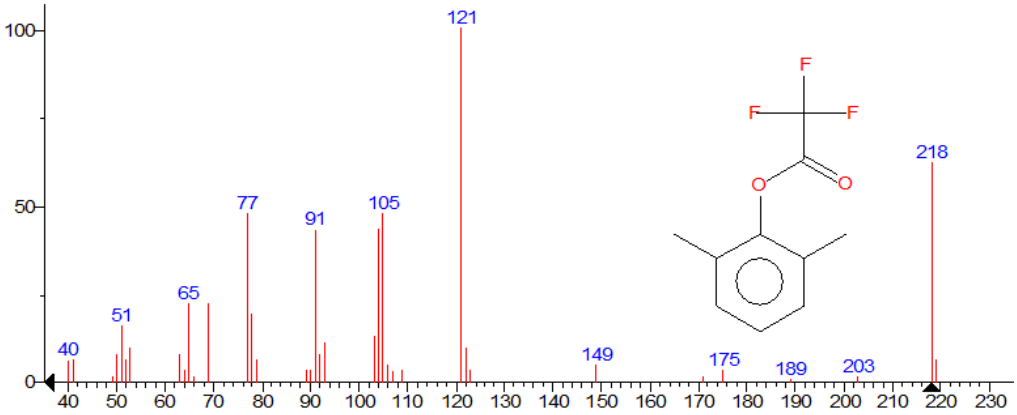
e 2,4-dimethylphenol



f [1] 2,5- dimethylphenol (the derivative was detected as 2,4-dimethylphenyltrifluoroacetate)



f [2] 2,5- dimethylphenol (the derivative was detected as 2,6-dimethylphenyl trifluoroacetate)



g 3,5- dimethylphenol

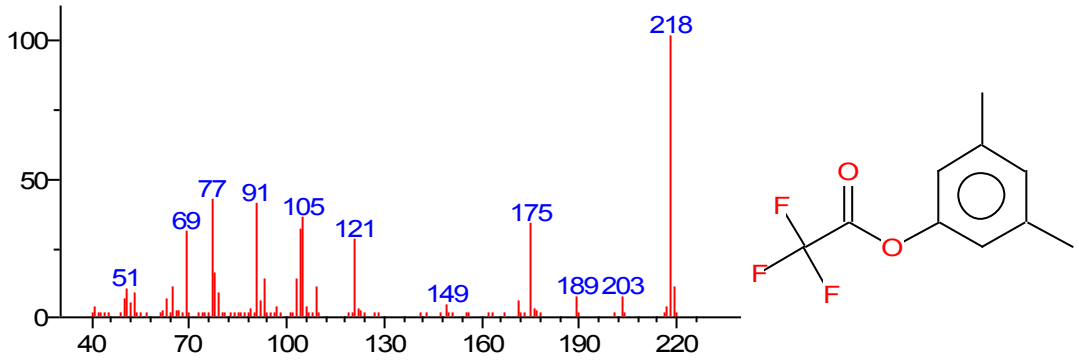
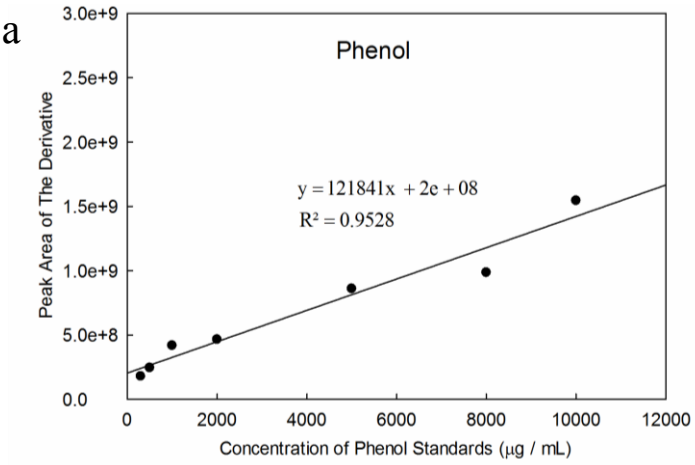
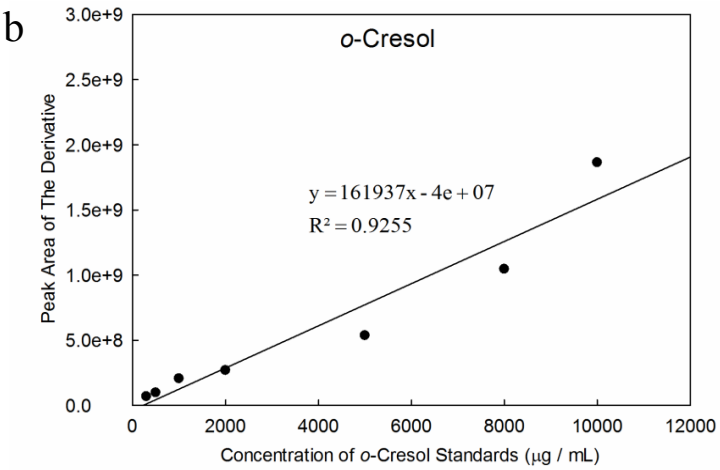


Figure 3.8 Mass spectra of phenols derivatized with TFAA. a. derivative of phenol; b. derivative of *o*-cresol; c. derivative of *m*-cresol; d. derivative of *p*-cresol; e. derivative of 2, 4-dimethylphenol; f. derivative of 2,5-dimethylphenol, the derivatives were always detected as a mixture of 2,4-dimethylphenyl trifluoroacetate and 2,6-dimethylphenyl trifluoroacetate; g. derivative of 3,5-dimethylphenol.

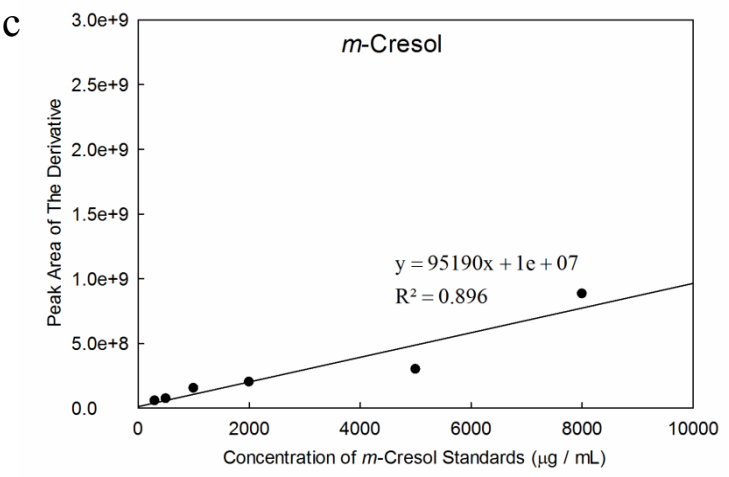
a



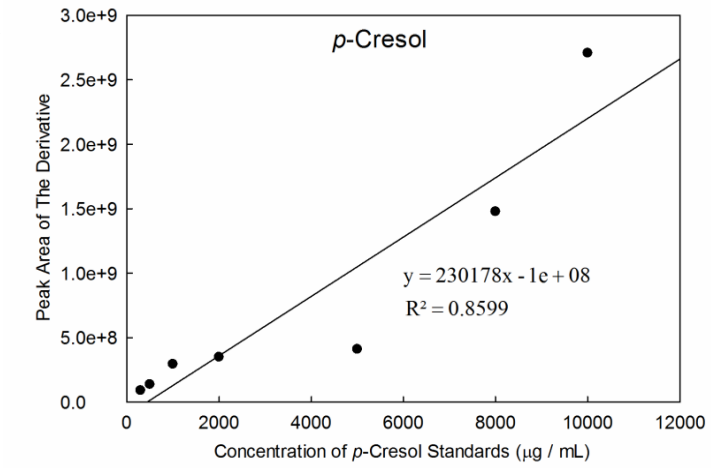
b



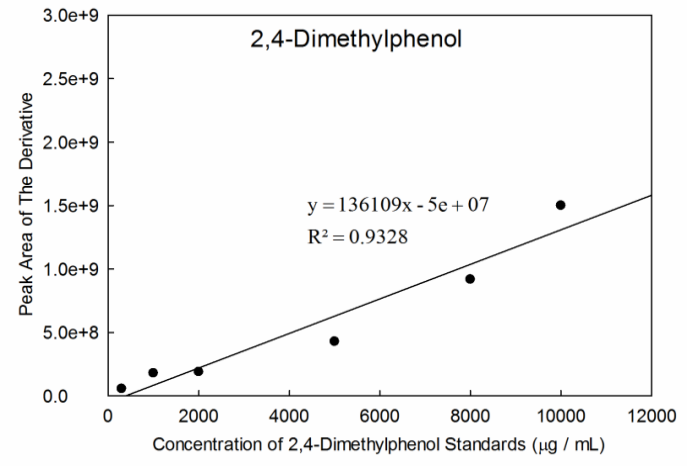
c


m-Cresol
y = 95190x + 1e + 07
R² = 0.896
Peak Area of The Derivative
Concentration of m-Cresol Standards (μg / mL)
3.0e+9
2.5e+9
2.0e+9
1.5e+9
1.0e+9
5.0e+8
0.0
0
2000
4000
6000
8000
10000

d


p-Cresol
y = 230178x - 1e + 08
R² = 0.8599
Peak Area of The Derivative
Concentration of p-Cresol Standards (μg / mL)
3.0e+9
2.5e+9
2.0e+9
1.5e+9
1.0e+9
5.0e+8
0.0
0
2000
4000
6000
8000
10000
12000

e


2,4-Dimethylphenol
y = 136109x - 5e + 07
R² = 0.9328
Peak Area of The Derivative
Concentration of 2,4-Dimethylphenol Standards (μg / mL)
3.0e+9
2.5e+9
2.0e+9
1.5e+9
1.0e+9
5.0e+8
0.0
0
2000
4000
6000
8000
10000
12000

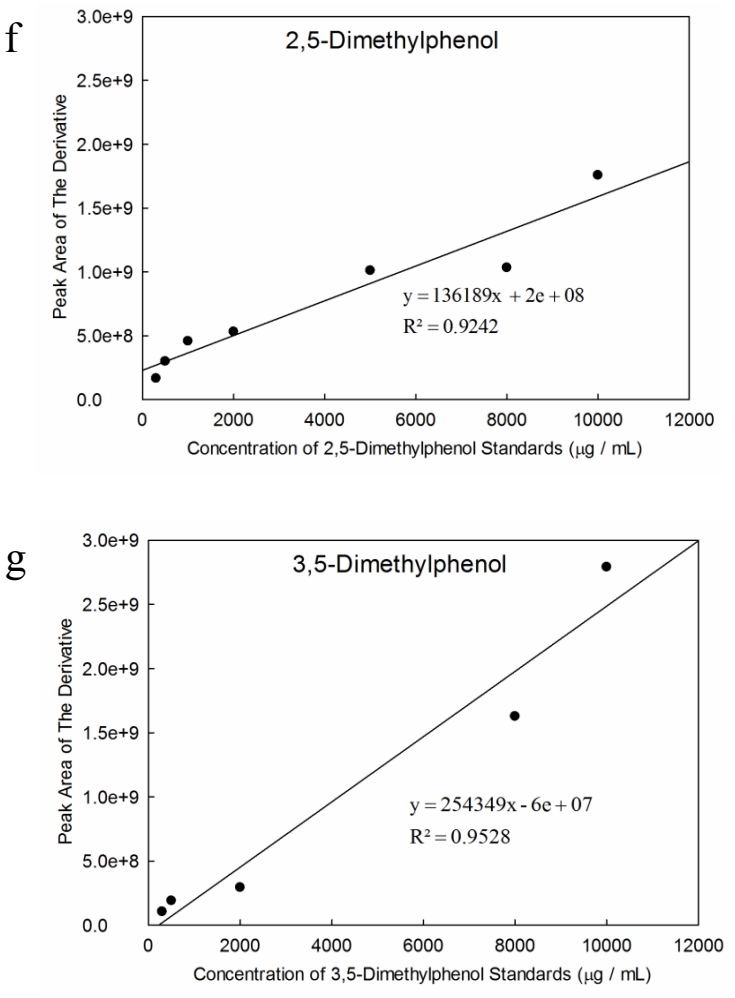


Figure 3.9 Calibration curve of the peak area of derivatives with its corresponding substrate standard solutions. a) Correlation of the peak area of phenyl trifluoroacetate to the concentration of phenol; b) – d) Correlation of the peak area of methylphenyl trifluoroacetate to the concentration of the respective cresol; e) – g) Correlation of the peak area of dimethylphenyl trifluoroacetate to the concentration of the respective dimethylphenols.

3.1.2.2 Method validation of phenols hydrogen isotope measurement by GC-IRMS

Standards of phenols were prepared in benzene. A lab standard of an *n*-alkane mix was analyzed before measurement and in between the measurement to ensure the data quality of the δ^2H values. All the acquired δ^2H values were calibrated using a two-point calibration with the *n*-alkane standards. δ^2H values of the phenols were calculated from the measured δ^2H values of the corresponding derivatives using the method described by Glaser & Gross (Glaser and Gross 2005). The Method Detection Limits (MDL) were determined and the required sample sizes for each compound are shown in Table 3.3. Comparing the results obtained by EA and GC-Cr/HTC-IRMS for standards, the highest offset in δ^2H values was observed for 2,4-dimethylphenol (24.2 ‰), and the lowest offset

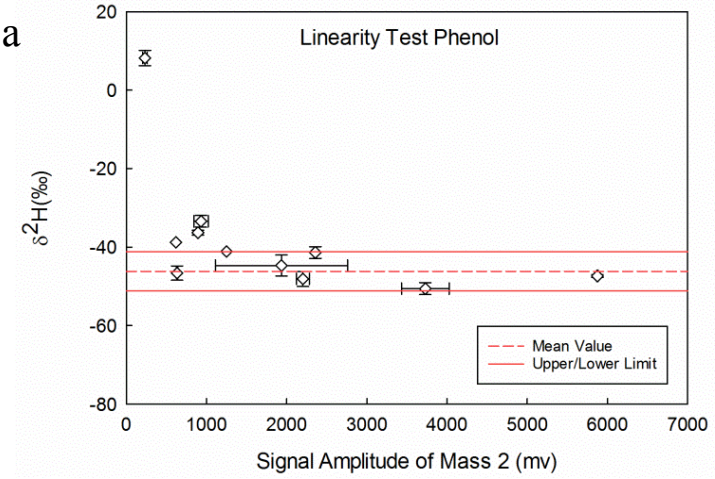
in δ^2H values was observed for 2,5-dimethylphenol (4.8 ‰). As a control, phenyl trifluoroacetate, which is the derivative of phenol by this method, was purchased as a pure compound and measured under the same conditions. The offset of δ^2H values obtained from EA and GC-Cr/HTC-IRMS for this compound was 7.5 ‰. A linear range is apparent for signals above the shown minimum amplitude (Figure 3.10). It is therefore concluded that the measurement method is valid. For each sample triplicate analyses were done. The precision of triplicate GC-IRMS measurements was below 5 ‰ for hydrogen isotopes.

Table 3.3 Method validation parameters for hydrogen stable isotopes of phenols

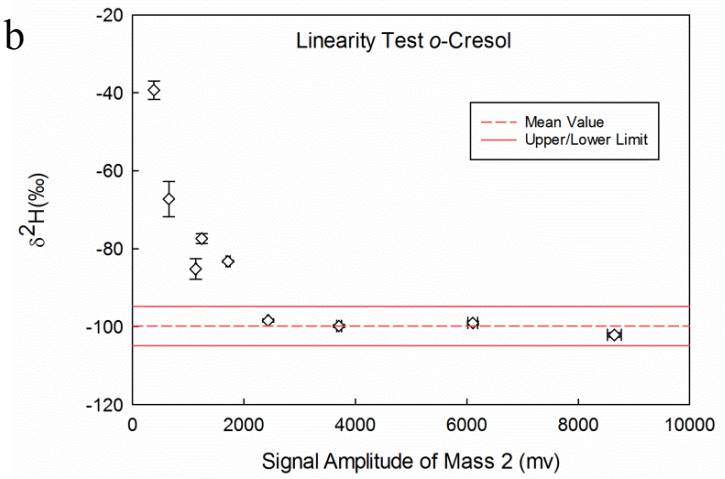
	MDL* (nmol of H on column)	MDL* (nmol)	Minimum peak amplitude of *m/z* 2 for accurate and precise isotope values [mV]	EA-IRMS value [‰]	GC-Cr/HTC-IRMS value [‰]	Isotopic shift [‰]
Phenol	52.6	10.5	1100	-24.1 ± 0.6	-38.5 ± 4.2	14.4
o-Cresol	85.8	12.3	2000	-71.1 ± 1.2	-87.4 ± 1.5	16.3
m-Cresol	54.9	7.8	1000	-105.1 ± 1.7	-111.4 ± 4.5	6.3
p-Cresol	54.9	7.8	1100	-116.3 ± 0.3	-123.6 ± 2.7	7.3
2,4-Dimethylphenol	41.3	4.6	1000	-191.2 ± 0.6	-167.0 ± 4.7	24.2
2,5-Dimethylphenol	165.1	18.3	3000	-112.7 ± 0.5	-117.5 ± 5.8	4.8
3,5-Dimethylphenol	41.3	4.6	1000	-109.7 ± 0.6	-126.1 ± 2.9	16.4
Phenyl trifluoroacetate	65.8	13.2	1000	-17.8 ± 1.3	-25.3 ± 2.3	7.5

* MDL: method detection limit. The second column shows the method detection limit of hydrogen atoms in phenols and the third column shows the method detection limit of the phenols.

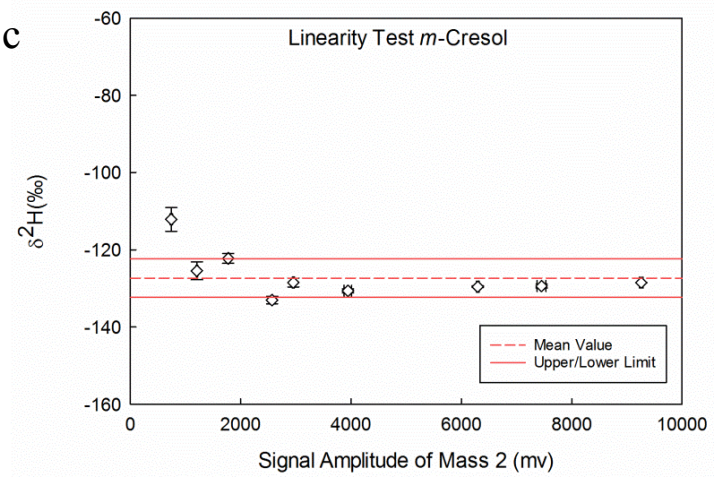
a


Linearity Test Phenol
δ2H(‰)
Signal Amplitude of Mass 2 (mv)
Mean Value
Upper/Lower Limit
20
0
-20
-40
-60
-80
0
1000
2000
3000
4000
5000
6000
7000

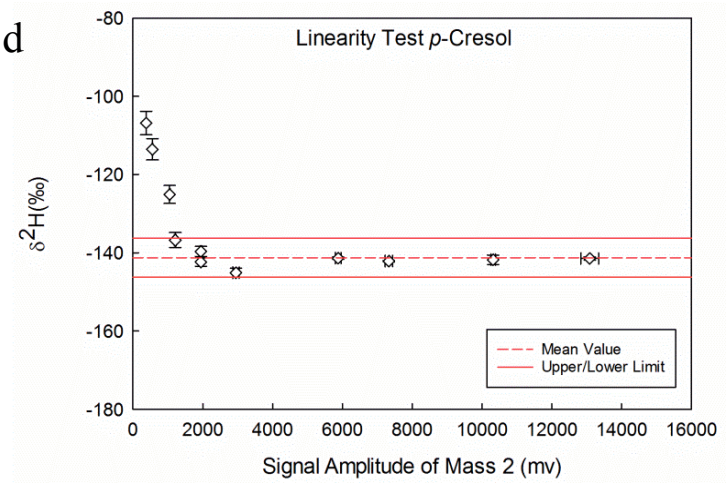
b


Linearity Test o-Cresol
δ2H(‰)
Signal Amplitude of Mass 2 (mv)
Mean Value
Upper/Lower Limit
-20
-40
-60
-80
-100
-120
0
2000
4000
6000
8000
10000

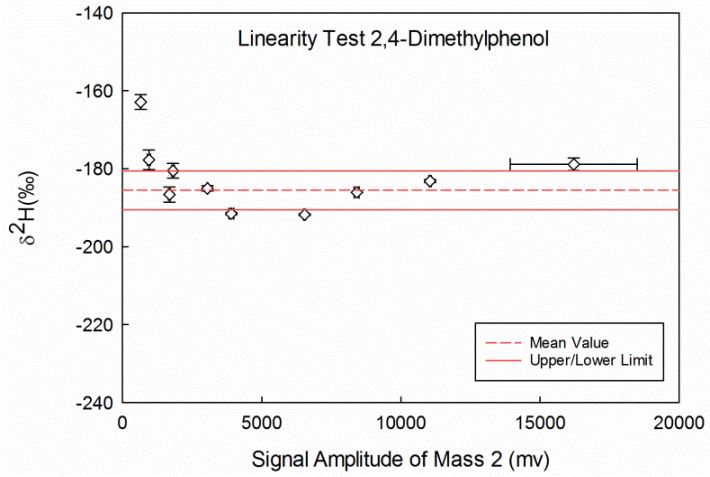
c


Linearity Test m-Cresol
δ2H(‰)
Signal Amplitude of Mass 2 (mv)
Mean Value
Upper/Lower Limit
-60
-80
-100
-120
-140
-160
0
2000
4000
6000
8000
10000

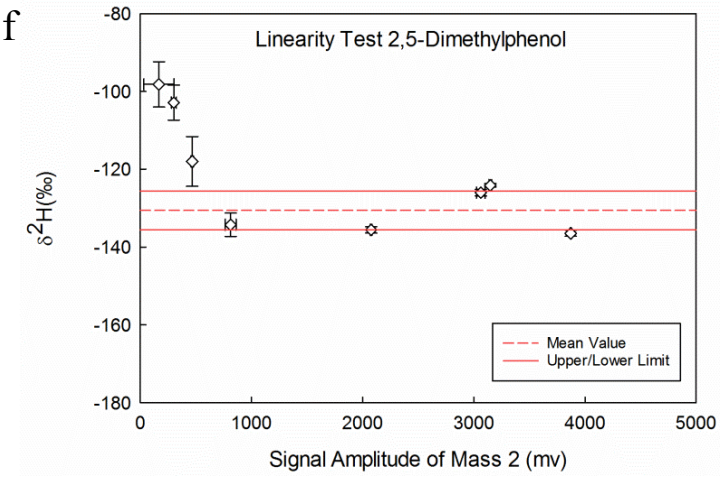
d


Linearity Test p-Cresol
δ2H(‰)
-80
-100
-120
-140
-160
-180
0
2000
4000
6000
8000
10000
12000
14000
16000
Signal Amplitude of Mass 2 (mv)
Mean Value
Upper/Lower Limit

e


Linearity Test 2,4-Dimethylphenol
δ2H(‰)
-140
-160
-180
-200
-220
-240
0
5000
10000
15000
20000
Signal Amplitude of Mass 2 (mv)
Mean Value
Upper/Lower Limit

f


Linearity Test 2,5-Dimethylphenol
δ2H(‰)
-80
-100
-120
-140
-160
-180
0
1000
2000
3000
4000
5000
Signal Amplitude of Mass 2 (mv)
Mean Value
Upper/Lower Limit

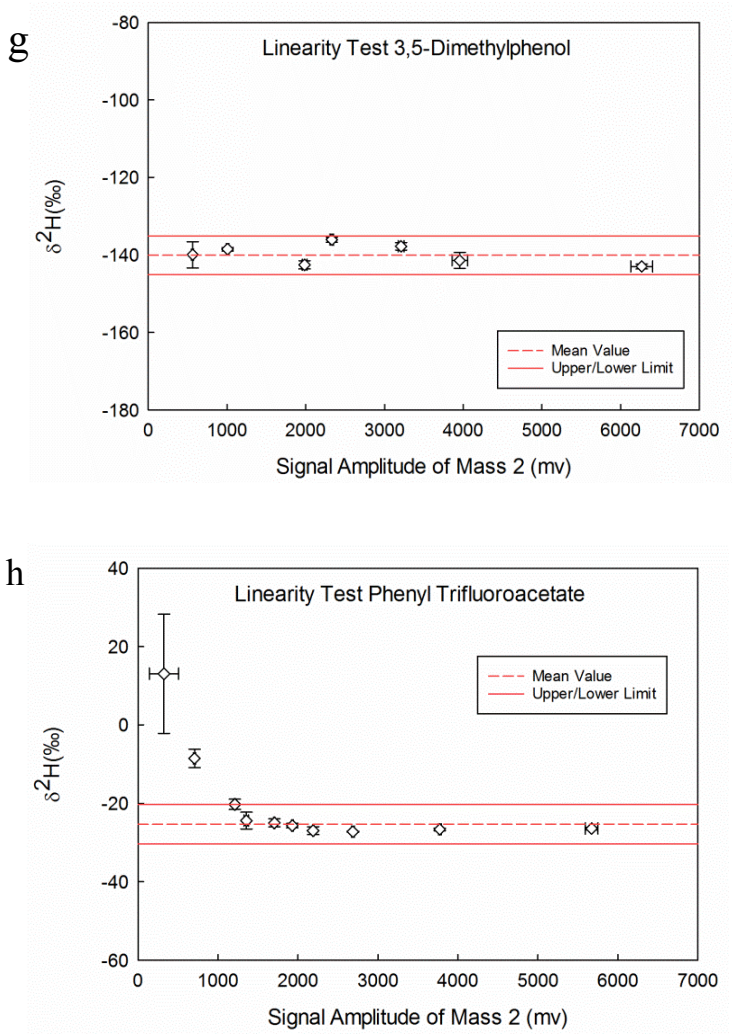


Figure 3.10 Representative calibration lines of phenols. The diamonds (◊) relate to δ^2H values. The red dashed line shows the δ^2H mean value. The red lines represent the interval of δ^2H mean value ± 5‰. a) Phenol; b) *o*-cresol; c) *m*-cresol; d) *p*-cresol; e) 2,4-dimethylphenol; f) 2,5-dimethylphenol; g) 3,5-dimethylphenol.

3.1.2.3 Hydrogen isotope fractionation upon biodegradation of *m*-cresol by *D.cetonica*

To verify the application of the developed hydrogen isotope analysis method for phenolic compounds, a biodegradation experiment was performed using *D.cetonica* cultivated under sulfate-reducing conditions amended with *m*-cresol as the sole source of carbon and energy. The substrate concentration was 0.5 mM and the completed degradation of *m*-cresol took about 7 days (Figure 3.11). The initial hydrogen isotope composition was of -113.8‰. A significant hydrogen isotope fractionation of 67.0‰ was detected, producing an enrichment factor of -96.4 ± 49.1‰ and an $AKIE_H$ value of 3.1 ± 1.5 (Figure 3.12).

Concentration decrease and changes in δ^2H values in abiotic controls were not observed (-116.4‰ at the beginning and -109.8‰ at the last time point), confirming that no other processes besides biodegradation caused the hydrogen isotope fractionation in the system.

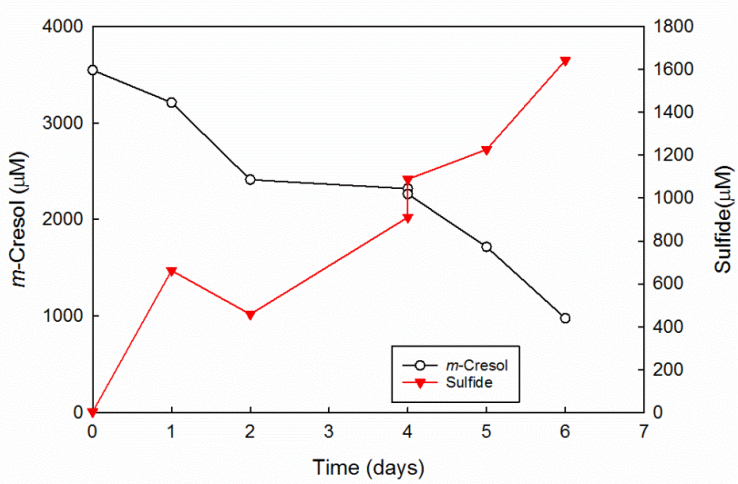


Figure 3.11 Degradation kinetics of *D.cetonica* for *m*-cresol as substrate (sulfate reducing conditions). Samples were used for hydrogen isotope analysis of m-cresol (see Figure 3.12).

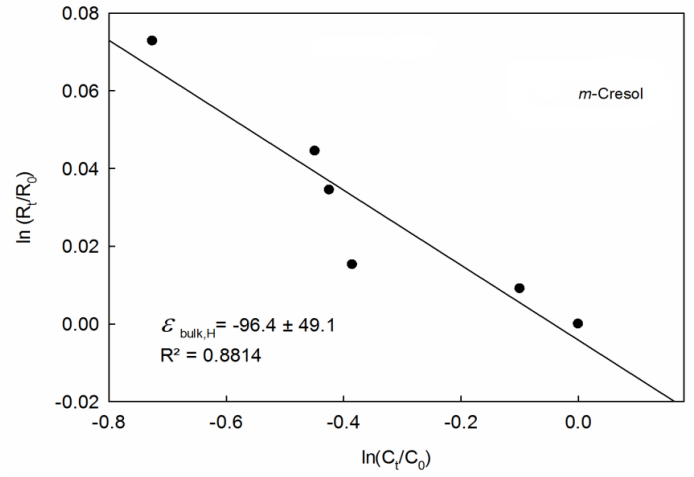


Figure 3.12 Rayleigh plot of hydrogen isotope fractionation for *D.cetonica* grown on *m*-cresol. Bulk enrichment factors (‰) were calculated from the slope of the regression curve and linearity is expressed in r^2 values.

3.1.3 Method development, validation and application of carbon and hydrogen isotope analysis of *n*-hexadecane

3.1.3.1 Method development and validation of *n*-hexadecane carbon and hydrogen isotope analysis by GC-IRMS

The procedures of method development and validation are described in chapter 2.3.3.6. The Method Detection Limits (MDL) were determined and the required sample sizes are shown in Table 3.4. Comparing the results obtained by EA and GC-IRMS for standards, an offset in $\delta^{13}C$ values of 0.3 ‰ was observed and an offset in $\delta^{2}H$ value of 27.4 ‰ was observed. A linear range is apparent for signals above the shown minimum amplitude (Table 3.4, Figures 3.13 and 3.14). The measurement method was therefore concluded as valid. Each sample was analysed in triplicate. The precision of triplicates GC-IRMS measurements was below ± 0.5 ‰ for carbon stable isotopes and below 5 ‰ for hydrogen stable isotopes.

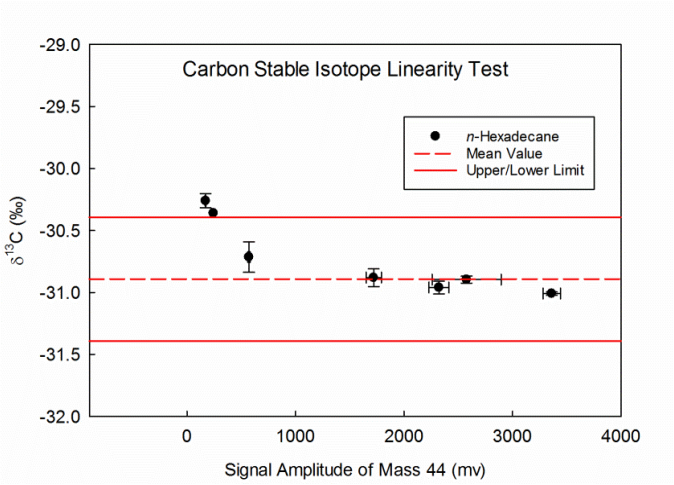


Figure 3.13 Representative calibration lines of carbon stable isotopes of *n*-hexadecane. The black dots relate to $\delta^{13}C$ values. The red dashed line shows the $\delta^{13}C$ mean value of all values with amplitudes above 1000. The red lines represent the interval of $\delta^{13}C$ mean value ± 0.5‰.

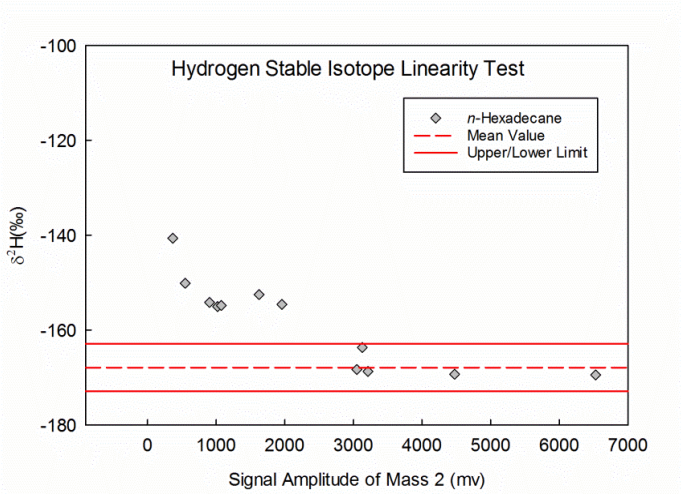


Figure 3.14 Representative calibration lines of hydrogen stable isotopes of *n*-hexadecane. The grey diamonds relate to δ^2H values. The red dashed line shows the δ^2H mean value of all values with amplitude above 3000. The red lines represent the interval of δ^2H mean value ± 5‰.

Table 3.4 Method validation parameters for carbon and hydrogen stable isotope of *n*-hexadecane

	MDL* (ng of C/H on column)	MDL* (nmol)	Minimum peak amplitude for accurate and precise isotope values [mV]	EA-IRMS value [‰]	GC- IRMS value [‰]	Isotopic shift [‰]
Carbon	76.8	0.4	570	-31.2 ± 0.1	-30.9± 0.1	0.3
Hydrogen	204.0	6.0	3000	-140.5 ± 0.8	-167.9 ± 2.4	27.4

MDL: Method detection limit. The second column shows the method detection limit of carbon/ hydrogen atoms in phenols and the third column shows the method detection limit of the phenols.

3.1.3.2 Carbon and hydrogen isotope fractionation upon biodegradation of *n*-hexadecane by *D.oleovorans* Hxd3

The study of isotope fractionation upon the degradation of *n*-alkanes has been mostly focused on short to medium chain *n*-alkanes (Kinnaman et al. 2007, Jaekel *et al.* 2014, Vogt *et al.* 2016). There is no isotope fractionation data available upon the anaerobic degradation of *n*-hexadecane. The sulfate-reducer *Desulfococcus oleovorans* has been identified as the first bacterium to degrade alkanes anaerobically (Aeckersberg *et al.* 1991, So *et al.* 2003). The degradation mechanism has been proposed to be a carboxylation at C3 position (So *et al.* 2003). Recently the group of Prof. Heider (Philipps University of Marburg) has proposed a new activation mechanism for the degradation, namely hydroxylation at C2 position. This part of the thesis aims at providing evidences for the activation mechanism from the aspect of isotope fractionations.

For the cultivation of *D.oleovorans* Hxd3, two methods for the addition of substrate were performed. In the first method, the *n*-hexadecane was directly injected into the cultures and the cultures were sacrificed at different time points with subsequently substrate extraction by *n*-hexane. Comparatively, in the other method, *n*-hexadecane was firstly dissolved in *n*-hexane and the mixture was added into the cultures. *n*-Hexane functions as

a carrier phase for the substrate, as *n*-hexane was known to be not degradable by *D.oleovorans*. However, with the carrier phase method, no degradation was observed.

Under the experimental conditions with the direct injection method, anaerobic biodegradation of *n*-hexadecane by *D.oleovorans* were monitored for 4 months; the substrates were completely degraded within 97 days (Figure 3.15). There was relatively high starting sulfide concentration in the cultures (1278.8 ± 80.8 µM) due to the high sulfide concentration in the pre-culture used for inoculation and the medium composition. There was however no significant increase in sulfide concentration, the sulfide concentration at the last sampling point was 1261.3 µM. This is probably due to sulfide precipitation with metal ions in the cultures as particles in black color were observed during the cultivation. No obvious enrichment in the ^{13}C value was observed. The initial carbon isotope composition of was -30.7‰ and when 96% of the substrate was depleted, the ^{13}C value was -30.4‰ (Figure 3.16). Therefore, an insignificant enrichment factor of -0.1 ± 0.098 was produced (Figure 3.17).

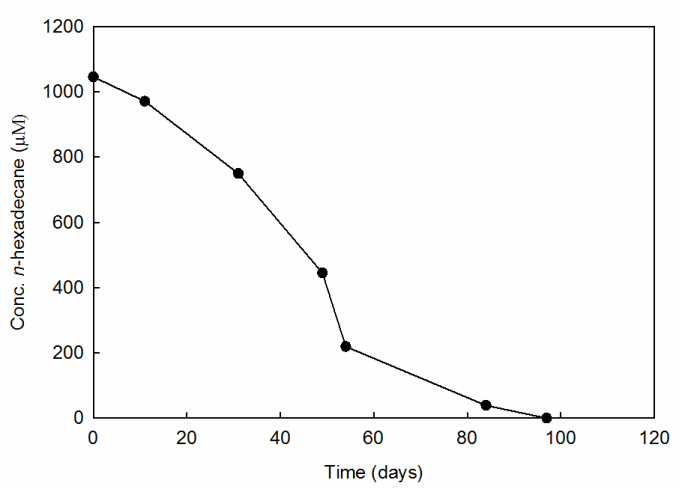


Figure 3.15 Degradation kinetics of *D.oleovorans* culture on substrate *n*-hexadecane.

For the measurement of hydrogen isotopic signatures, valid values were only obtained for the first two sampling points during the degradation process, namely -130.8 ± 0.9 ‰ for the initial value and -111.2 ± 0.0 ‰ when around 8% of the substrate was degraded. The remaining measurement values were discarded due to the lower signal amplitude than the method detection limit. The bottleneck here was the low extraction efficiency with this method. The concentration of *n*-hexadecane in the extracts of the other time points was lower than the MDL for hydrogen isotope analysis.

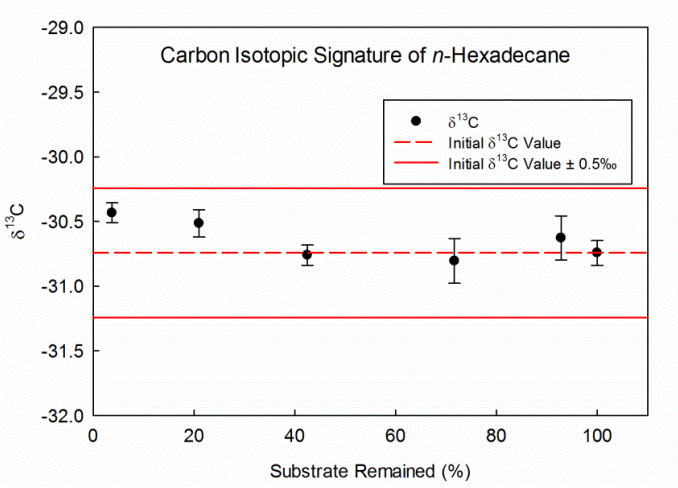


Figure 3.16 Comparison of carbon isotopic signature of *n*-hexadecane at different sampling points. Black dots represent the $\delta^{13}C$ values. Red dash line is the $\delta^{13}C$ value of the initial composition of *n*-hexadecane and the red lines represents the interval of initial $\delta^{13}C$ value ± 0.5 ‰.

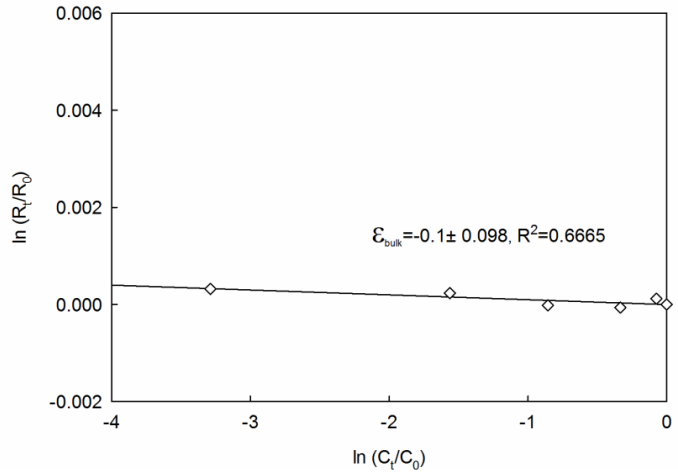


Figure 3.17 Rayleigh plots of carbon isotope fractionation for *D.oleovorans* grown on *n*-hexadecane. Bulk enrichment factors (‰) were calculated from the slope of the regression curve and linearity is expressed in r^2 values.

3.2 Identification of anaerobic ethylbenzene-assimilating organisms by SIP and other approaches

A strictly anoxic ethylbenzene degrading enrichment cultures obtained from a contaminated aquifer in Zeitz, Germany, was investigated in more detail. Ethylbenzene degradation was coupled to sulfide production, demonstrating that sulfate is used as terminal electron acceptor for ethylbenzene degradation. Illumina sequencing and metaproteomics analysis were performed with DNA extracted from the enrichment

culture. A DNA stable isotope probing experiment (DNA-SIP) was performed with ring-labeled ^{13}C-ethylbenzene as substrate to unravel the microorganisms assimilating carbon from ethylbenzene in the sulfate reducing enrichment culture. The growth of the cultures and the accumulation of $^{13}CO_2$ in the headspace of the cultures were monitored for over 500 days. Due to the extremely slow mineralization rate, a protein analysis was performed instead of DNA analysis for the labeled culture in the end.

3.2.1 Growth kinetics of the Zeitz ethylbenzene-degrading enrichment culture

The enrichment of active sand from Zeitz was attempted with ethylbenzene as electron donor and sulfate as electron acceptor. The samples were taken from the sand-filled columns flushed on site with BTEX-containing sulfidic groundwater as described in chapter 2.6.1 and four bottles of 1 liter enrichment culture were set up as described in chapter 2.2.7. The goal was to establish an anaerobic ethylbenzene-degrading enrichment culture under sulfate-reducing condition for the further identification of the ethylbenzene assimilating organisms. Generally, the cultivation of ethylbenzene-degrading bacteria is demanding and only a few cultures have been described under sulfate-reducing condition (Elshahed et al. 2001, Nakagawa et al. 2002, Weelink *et al.* 2010). No enrichment culture from fresh water environment under this condition has been hitherto described.

The degradation was monitored by the measurement of ethylbenzene concentration in the culture and the accumulation of sulfide. Overall, the degradation of around 0.3 mM ethylbenzene took 200-300 days (1.62 µM / day on average) with significant sulfide accumulation. The degradation kinetics of two enrichment cultures, which was used for further analysis, is shown in Figure 3.18.

Attempts to accelerate the mineralization process were tried by setting up cultures with additional yeast extract (0.05%) in the medium. A significant increase in sulfide production was observed in these cultures while the degradation of ethylbenzene was not obviously accelerated. This indicates that yeast extract was used as additional carbon source disturbing the enrichment process in this way.

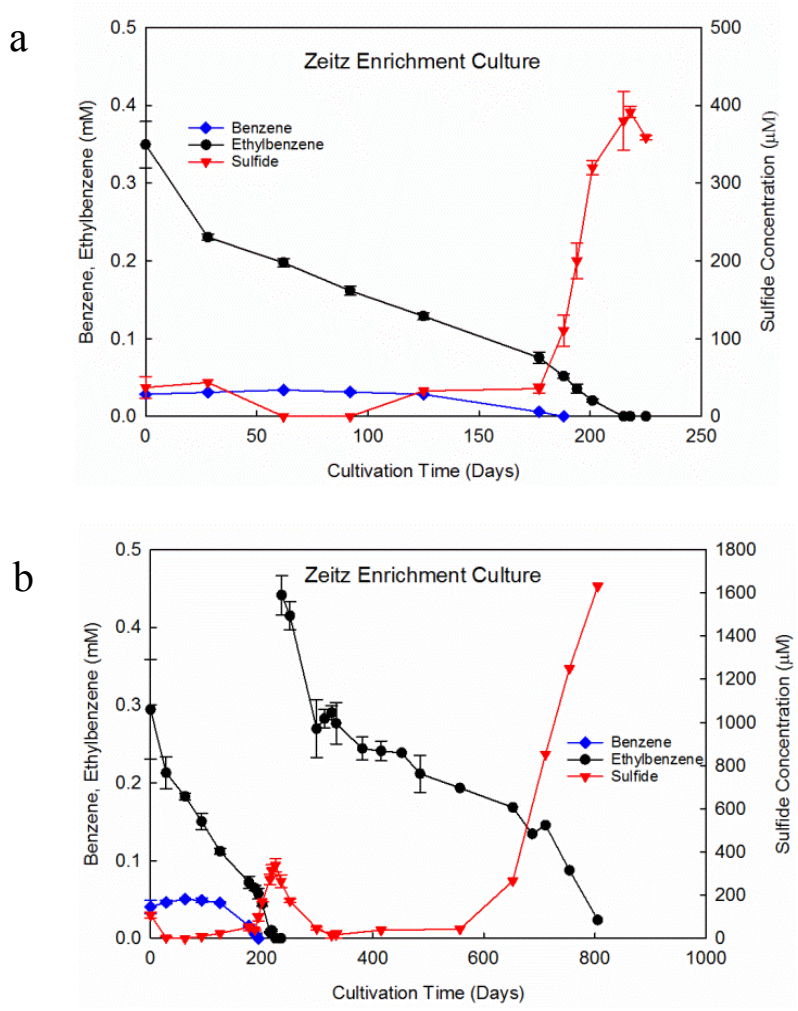


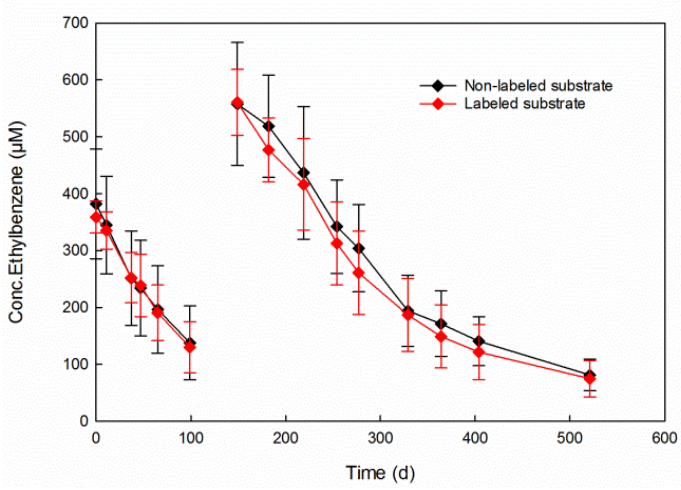
Figure 3.18. Degradation kinetics of ethylbenzene-degrading enrichment cultures. Benzene originated from the contaminated groundwater used to set up the enrichment cultures. Culture a) was cultivated till the substrate was completely degraded and was further used for the SIP experiment. Culture b) was spiked twice with ethylbenzene and at the end of the degradation (shortly after 800 days) was sacrificed for metaproteomics analysis.

3.2.2 Stable isotope probing (SIP) experiments with the Zeitz enrichment culture

Eighteen individual microcosms were set up from one active enrichment culture (growth kinetics shown in Figure 3.18, a) as described in chapter 2.2.9. Nine microcosms were added with non-labeled ethylbenzene while the other nine were spiked with ring labeled ^{13}C ethylbenzene to final substrate concentrations of around 0.4 mM. Four other microcosms served as abiotic controls with either labeled or non-labeled ethylbenzene as substrate and two microcosms as ethylbenzene-free controls. The average degradation rate for cultures grown on non-labeled ethylbenzene was 1.38 µM / day and for cultures

grown on labeled ethylbenzene was 1.37 µM / day (Figure 3.19a). The degradation rate between microcosms grown on labeled or non-labeled substrate did not show any difference and was hence similar. The sulfide concentration increase was not significant which was probably caused by the slow degradation process and the formation of precipitation with sulfide and other ions such as Fe^{2+} in the cultures (Figure 3.19b). There was decrease in ethylbenzene concentration in abiotic controls at a similar rate to the biotic microcosms which might be explained by absorption to the sand (Figure 3.20a). No sulfide accumulation was observed in either abiotic or substrate-free controls (Figure 3.20b). Due to the considerable and unexpected losses of ethylbenzene in the abiotic controls, the evaluation of the amount of biodegraded ethylbenzene by analyzing the concentration decrease was difficult. The measurement of the produced $^{13}CO_2$ however allowed estimating how much ethylbenzene was mineralized. The microcosms amended with ^{13}C-labeled ethylbenzene as growth substrate produced ^{13}C-labeled CO_2 in isotope signatures with an average value of $\delta^{13}C = +182.8 \pm 85.3$ ‰ (Figure 3.21), corresponding to 95.9 ± 28.2 µM produced CO_2 and 16.0 ± 4.7 µM (Figure 3.22) mineralized ethylbenzene. The production of $^{13}CO_2$ was already detected by the first sampling point, which was 45 days after the set-up, indicating the degradation without lag-phase after the addition of the substrate and the continuous accumulation indicating the continuous mineralization of the substrate. In the microcosms with non-labeled substrate, the carbon isotope signature did not change significantly (Figure 3.21) during the degradation process.

a



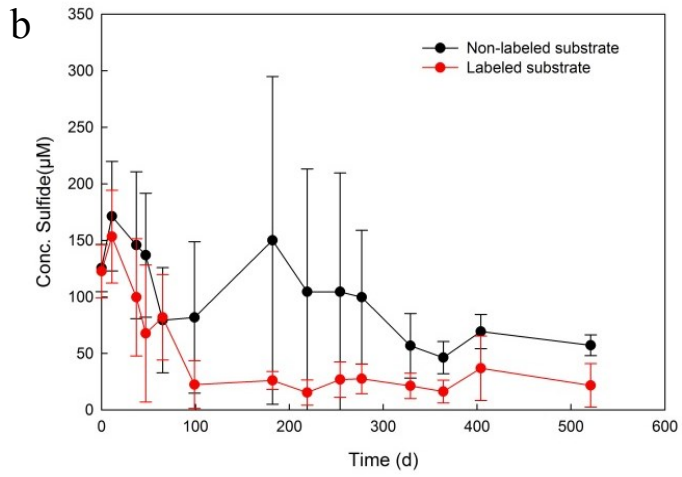
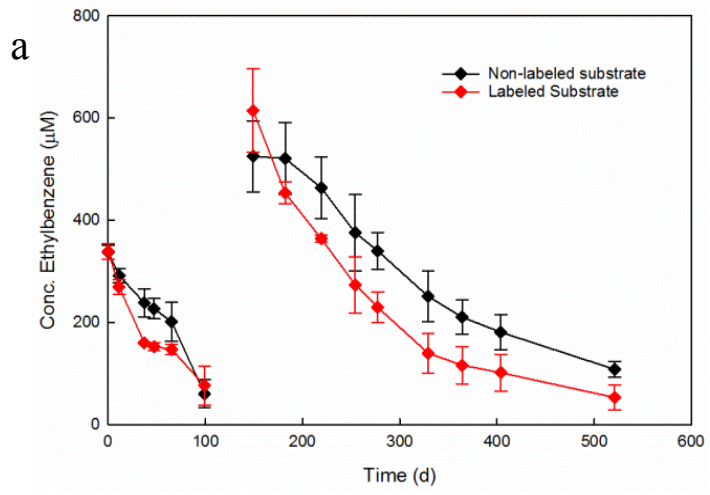


Figure 3.19 Degradation kinetics of the Zeitz ethylbenzene-degrading microcosms used for SIP. The figure is based on average values of all eplicates. a) Ethylbenzene concentration change. b) Sulfide concentration change.



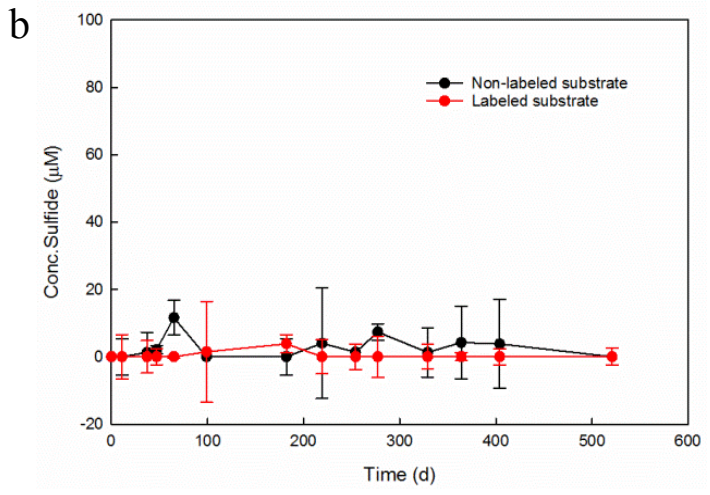


Figure 3.20 Ethylbenzene and sulfide concentration changes in the control cultures of the Zeitz

stable isotope probing experiment. The figure is based on average values of all the replicates. a) Ethylbenzene concentration change. b) Sulfide concentration change.

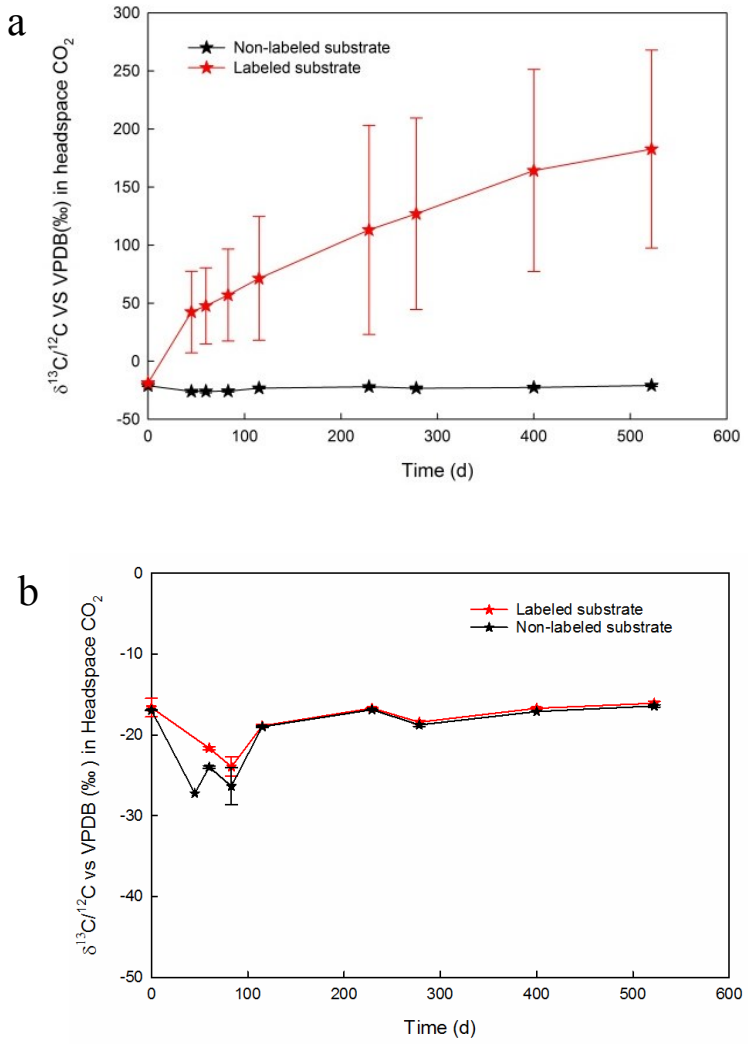


Figure 3.21 (a) The enrichment of $^{13}CO_2$ in the headspace of microcosms grown on labeled and non-labeled ethylbenzene. (b) The $^{13}CO_2$ signature in the headspace of abiotic control cultures. The figure is based on average values of all replicates.

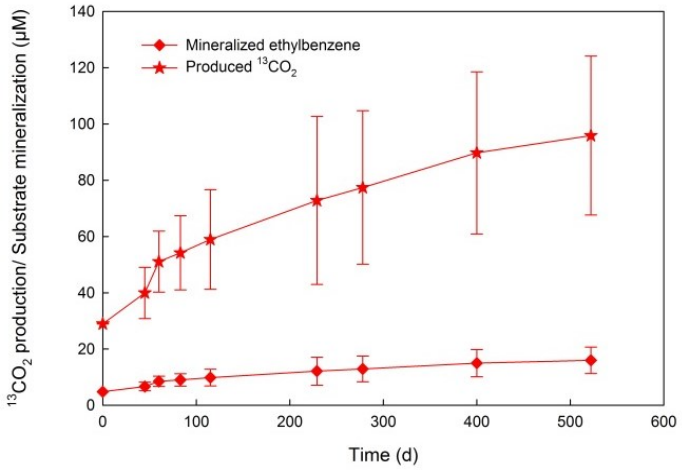


Figure 3.22 Production of ^{13}C-enriched CO_2 in microcosms grown on labeled ethylbenzene and the corresponding amount of mineralized ethylbenzene. The figure is based on average values of all replicates.

The slow mineralization rate indicated that the cells assimilated only low amounts of labelled carbon, a subsequent DNA analysis as planned was not meaningful because the separation of labeled and non-labeled DNA during ultracentrifugation requires a minimum of 30 atom % of ^{13}C incorporation (Jehmlich *et al.* 2008). Therefore, the cultures were used for a protein-SIP analysis which has higher sensitivity. Jehmlich and colleagues reported that a clear difference in the isotopic signatures of peptides can be detected in the incorporation of 1-2 atom % ^{13}C. Furthermore, proteins have a higher concentration in cells compared to DNA (Jehmlich et al. 2008). The analysis was performed by the Department of Molecular System Biology, Helmholtz Institute for Environmental Research, Leipzig, Germany. However, due to the low biomass, few peaks were identified and the Relative Isotope Abundance (RIA) could not be calculated by the pipeline.

3.2.3 Overall sequencing results of the enrichment culture

Two samples from two separate bottles of ethylbenzene-degrading, sulfate-reducing enrichment cultures as well as one benzene-degrading, sulfate-reducing enrichment culture from Zeitz were used for sequencing. The degradation kinetics of the two ethylbenzene-degrading cultures is shown in Figure 3.23. The dataset obtained from the Illumina Pipeline was initially processed by the working group of Professor Dietmar Pieper, Helmholtz Center for Infection Research, Braunschweig, Germany. A total number of 64,865 reads were obtained for three sequenced samples. 22366 and 21550 reads for the two samples of the ethylbenzene-degrading, sulfate-reducing cultures and 20949 reads for one sample from the benzene-degrading, sulfate-reducing culture which served as control and comparison in this study. The enrichment culture with benzene as substrate originated from the same system with those ethylbenzene-degrading cultures. The community structure in the benzene-degrading cultures have been studied (Herrmann et al. 2008). The dataset was then filtered to consider those phylotypes that were present in at least one sample at a relative abundance >0.1%. A total of 160 phylotypes could be resolved for further analysis. All phylotypes remained were assigned a taxonomic affiliation by alignment against the RDP database (Wang et al. 2007). An operational

taxonomic unit (OTU) is referred here as a sequence which shares at least 95% sequence identity with known 16S rRNA sequences; the number of OTUs refer to the relative abundance of sequences that were assigned for a taxon. The sequencing data are shown in Figure 3.24.

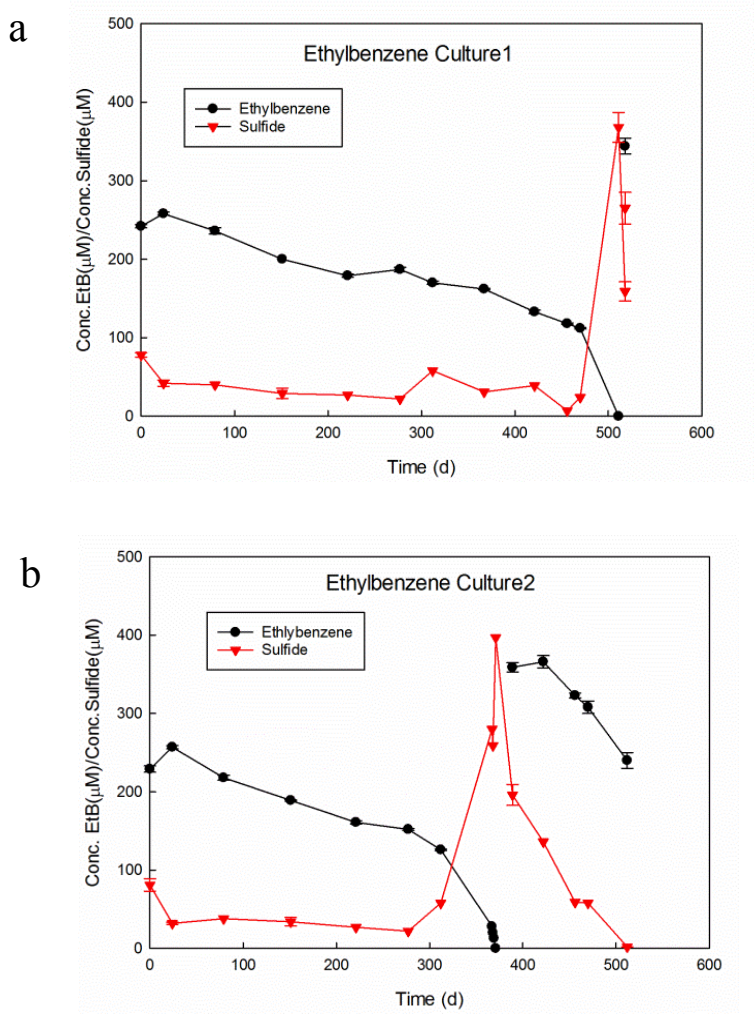


Figure 3.23 Degradation kinetics of Zeitz ethylbenzene-degrading cultures from which samples for Illumina sequencing were taken.

The microbial communities in the Zeitz enrichment culture were mainly dominated by three phyla: Chloroflexi, Proteobacteria and Parcubacteria (Candidate OD1). Other phyla with significant relative abundance were: Verrucomicrobia and Candidate division WPS-2. Members of these phyla were identified from sources such as fresh water, sediments and soil. In comparison with the benzene-degrading cultures, there is much lower presence of Firmicutes in both ethylbenzene-degrading cultures. The classes and genus

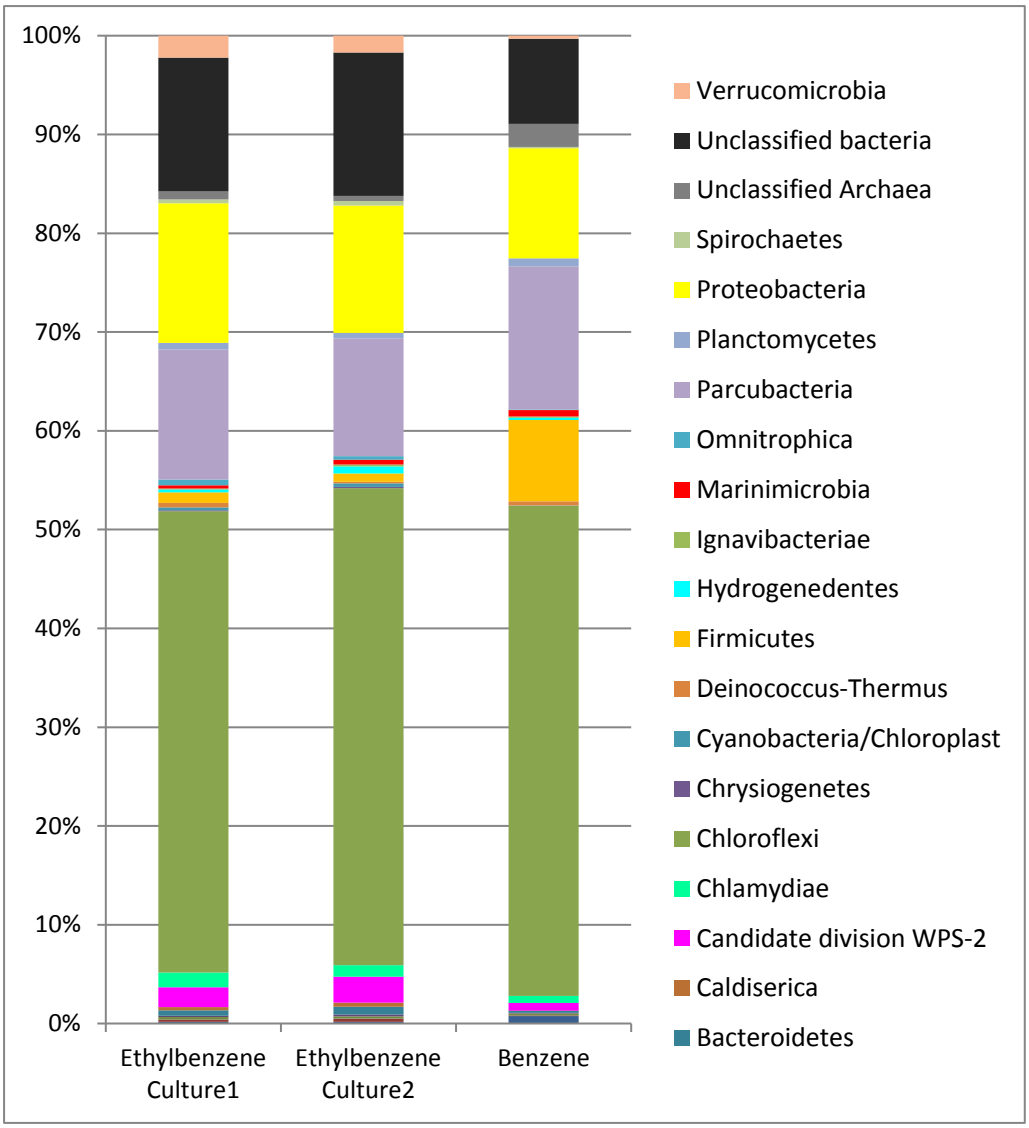
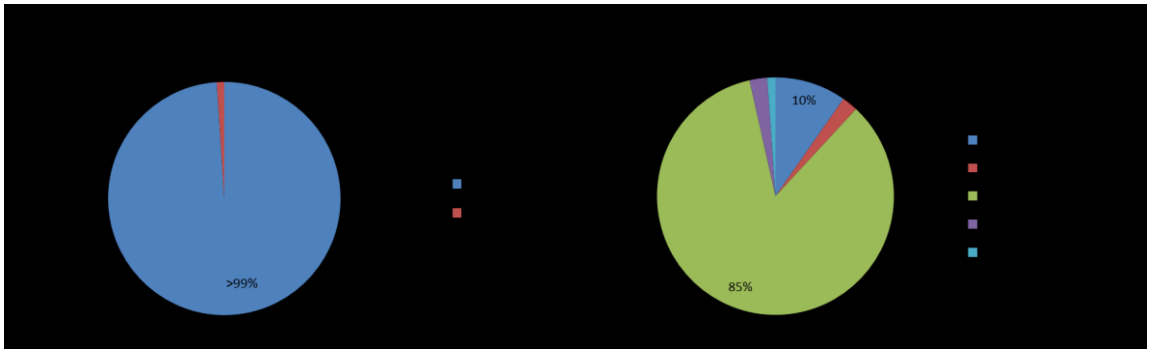
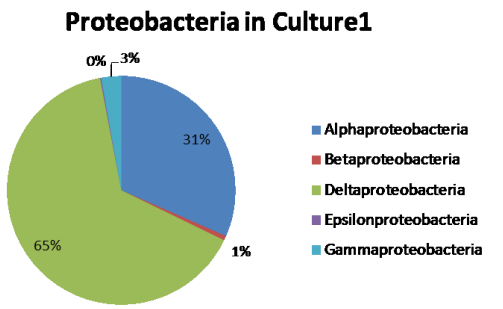


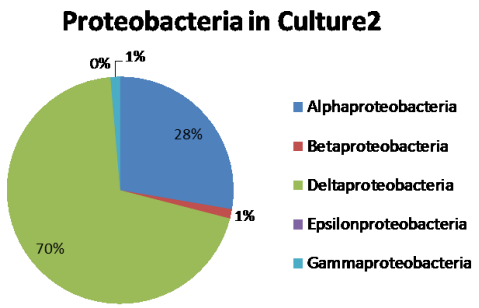
Figure 3.24 Relative abundances of phyla in sulfate-reducing, ethylbenzene-degrading enrichment cultures and a benzene-degrading reference culture.

within the dominant phyla that could be identified in both cultures were summarized in Figure 3.25. Over 99% of the phylum Chloroflexi in both cultures was dominated by the order Anaerolineales while the phlylum Parcubacteria was solely composed of the genus *Parcubacteria_genera_incertae_sedis*. Proteobacteria in both cultures consisted of mainly Alphaproteobacteria and Deltaproteobacteria. The orders Desulfobacterales and Syntrophobacterales occupied more than 95% of the Deltaproteobacteria which was the main class within the phylum Proteobacteria in the two cultures. Betaproteobacteria, Epsilonproteobacteria and Gammaproteobacteria made up less than 5% of the whole Proteobacteria. Betaproteobacteria contained only the order Burkholderiales and

Epsilonproteobacteria contained only the order Campylobacterales while Gammaproteobacteria were composed of Pseudomonadales and Xanthomonadales. Proteobacteria in the culture grown on benzene consisted of mainly Alphaproteobacteria and Deltaproteobacteria as well. The orders Desulfobacterales (38.3%), Desulfovibrionales (32.3%) and Syntrophobacterales (23.9%) occupied over 90% of the Deltaproteobacteria. In comparison with the two cultures grown on ethylbenzene, there is a much higher percentage of Desulfovibrionales and a lower percentage of Syntrophobacterales in the benzene-degrading culture. The further detailed classification of orders in Alphaproteobacteria and Deltaproteobacteria is shown in Figure 3.26.







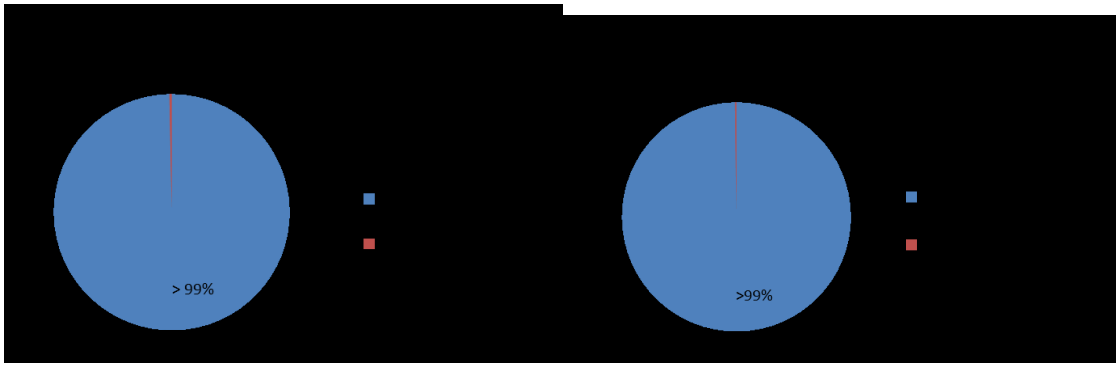
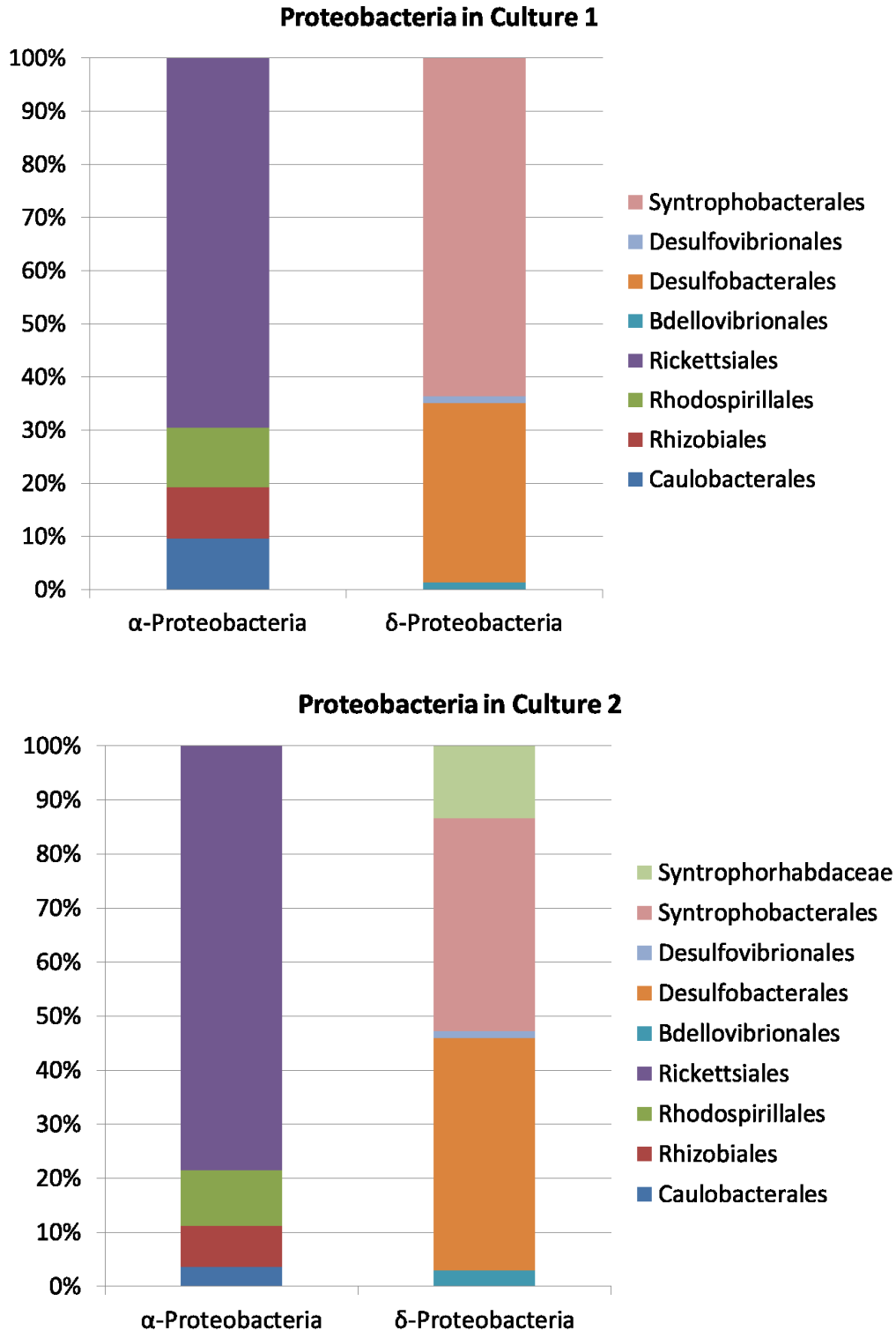


Figure 3.25 Relative abundance of classes/genus in the most abundant phyla in sulfate-reducing, ethylbenzene-degrading enrichment cultures. For comparison, the original culture grown on benzene is also shown.



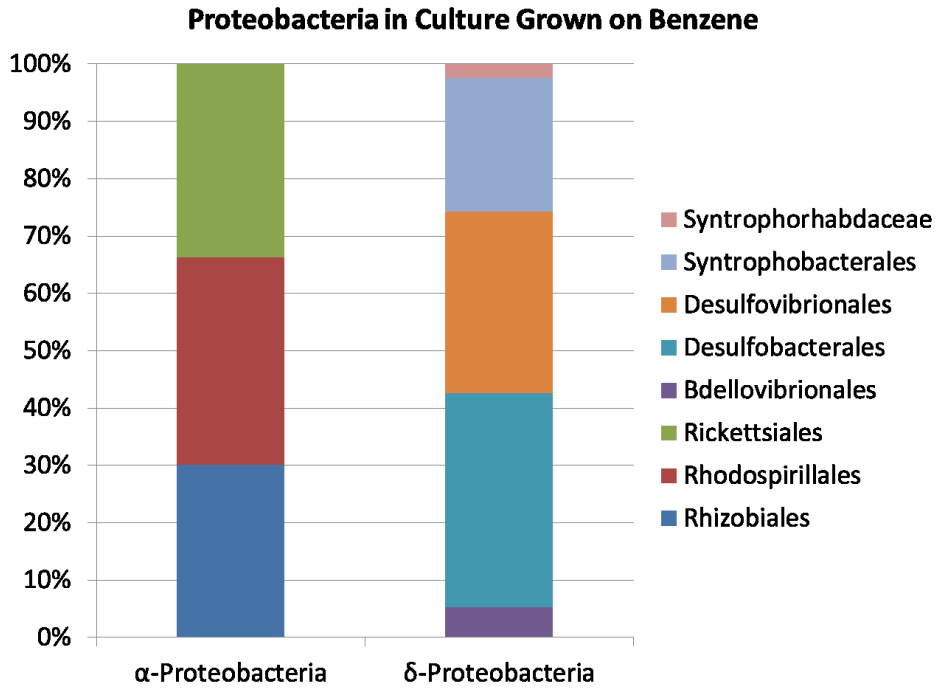


Figure 3.26 Relative abundance of orders in Alphaproteobacteria and Deltaproteobacteria in sulfate-reducing, ethylbenzene-degrading enrichment cultures and the culture grown on benzene.

3.2.4 Metaproteomics of the enrichment culture

The identification of ethylbenzene degraders with protein-SIP approaches has failed due to the very low biomass in the enrichment culture. To gain further information of the microbial community in the ethylbenzene-degrading culture, metaproteomics analysis of the culture was performed.

The degradation kinetics of the enrichment culture sacrificed for metaproteomics analysis is shown in Figure 3.18(b). The analysis was performed by the Department of Molecular System Biology, Helmholtz Center for Environmental Research, Leipzig, Germany. The data were obtained as annotated files in respect to the function and phylogenetic affiliation of the protein sequences. The contigs were regarded as identified with at least one unique peptide with false discovery rate < 1%. A total number of 379 identified protein sequences were assigned on basis of a metagenome dataset from the Zeitz contaminated aquifer. The metagenome dataset was constructed by combining the blastn and blastp results (blast: National Library of Medicine, Bethesda, MD, USA). The method is described in detail by Taubert and colleagues (Taubert *et al.* 2012). An overview of the phylogenetic classification on the level of phylum is shown in Figure 3.27. The Firmicutes and Proteobacteria were the most abundant phyla followed by

Euryarchaeota and Chloroflexi with significant abundance. On the level of class, within the phylum of Proteobacteria, Deltaproteobacteria (Figure 3.28) presented with significant abundance. It was mainly composed of the orders Syntrophobacterales, Desulfuromonadales and Desulfobacterales. Within the phylum Firmucutes, the Clostridiales showed over 90% abundance. The metabolic pathways of the ethylbenzene-degrading community identified by the metaproteome analysis are summarized in Table 3.5. Enzymes related to aromatic compound degradation were affiliated to the orders Clostridiales, Desulfuromonadales, Rhodocyclales and Rhodobacterales. While enzymes related to the dissimilatory sulfate reduction were mainly affiliated to the orders Clostridiales, Syntrophobacterales, Thermoanaerobacterales and other unidentified or unclassified bacteria. Other important metabolic function such as ATP synthesis were mainly assigned to the orders Clostridiales, Syntrophobacterales, Thermomicrobiales, Campylobacterales and other unidentified bacteria. The CO_2 fixation function was mainly assigned to the Clostridiales and Euryarchaeota.

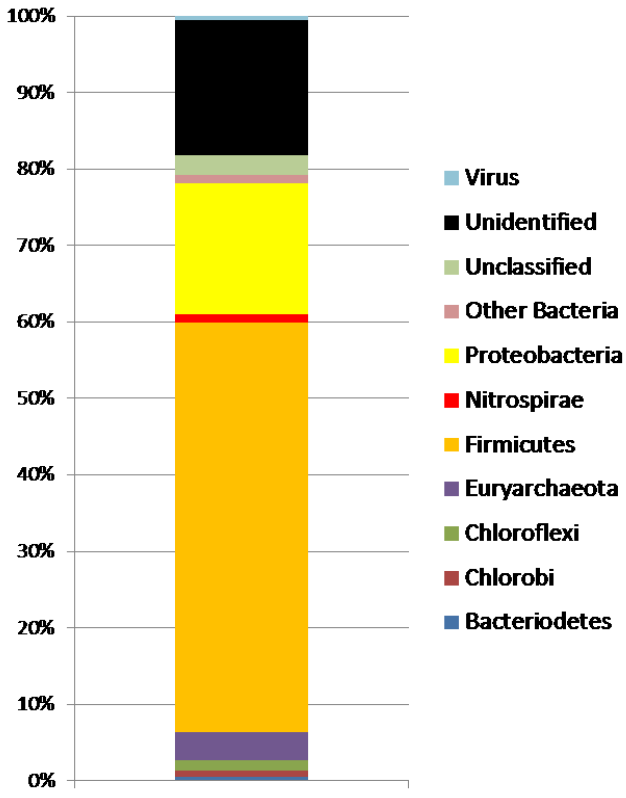


Figure 3.27. Relative abundance of the 379 identified protein sequences assigned to phyla.

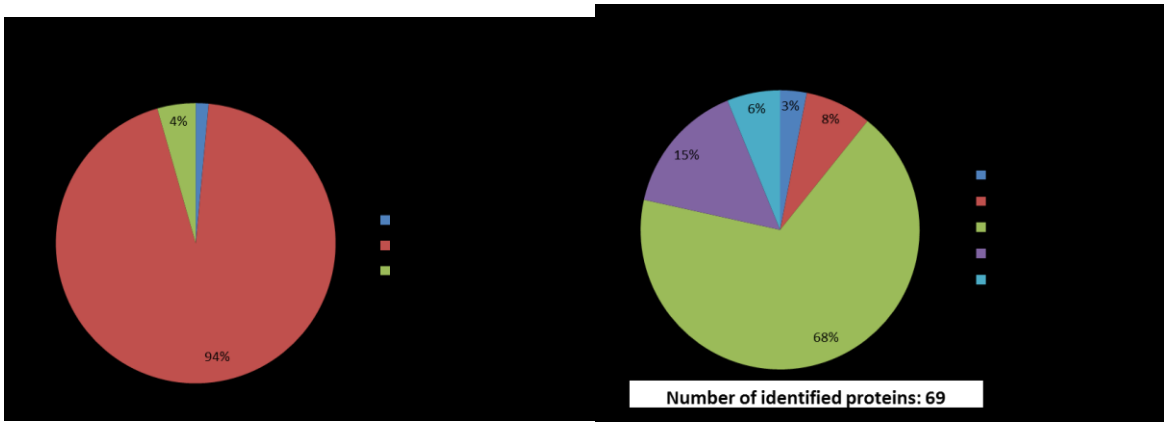


Figure 3.28 Relative abundance of class/genus in the two dominated phyla in sulfate-reducing, ethylbenzene-degrading enrichment culture.

Table 3.5 Functional classification of identified proteins

	In total	Firmicutes	Proteobacteria	Chloroflexi	Unidentified/ Unlassified	Other microbes
Aromatic compound degradation	9	3	5	0	0	1
ATP synthesis	14	5	4	2	3	0
Biomolecule synthesis and other metabolic process	57	48	4	0	0	5
C1 metabolism, CO_2 fixation	14	10	1	0	0	3
Cellular process, protein-interaction, regulation	49	21	8	2	16	2
Chaperonins	10	7	2	0	0	1
Conserved hypothetical protein	47	30	7	1	6	3
Dissimilatory sulfate reduction	17	8	2	0	7	0
Energy metabolism	52	31	11	1	3	6
Protein of unknown function	63	16	12	0	28	7
Transport/Binding	24	18	4	0	1	1
Unique hypothetical protein	23	6	9	0	4	4

The functional group aromatic compound degradation, dissimilatory sulfate reduction and C1 metabolism/CO2 fixation are the most important groups to understand the ethylbenzene degradation mechanism in the enrichment culture. The proteins within these groups are listed in Table 3.6.

Table 3.6 Identified proteins related to functional categories

Accession No.	Sequence ID	Description	Size (kDa)	Score	Coverage	Phylogeny (class)	Phylogeny (order)
Aromatic compound degradation							
ZBCMv2_5120001	ID:5766156	bamB| tungsten containing benzoyl-CoA reductase, aldehyde ferredoxin oxidoreductase	76.01	27.19	13.36	Proteobacteria	Desulfuromonadales
ZBCMv2_34560001	ID:5772089	bzdZ| 3-oxoacyl-acyl-carrier-protein] reductase	28.75	64.37	58.58	Proteobacteria	Rhodocyclales
ZBCMv2_15020002	ID:5768623	CoB--CoM heterodisulfide reductase (fragment)	23.17	13.48	11.57	Firmicutes	Clostridiales
ZBCMv2_12330001	ID:5768030	CoB--CoM heterodisulfide reductase iron-sulfur subunit A 2 (fragment)	33.23	36.87	35.83	Firmicutes	Clostridiales
ZBCMv2_7620003	ID:5766884	had| 6-hydroxycyclohex-1-ene-1-carbonyl-CoA dehydrogenase (fragment)	11.27	7.08	32.32	Proteobacteria	Rhodocyclales
ZBCMv2_16090001	ID:5768841	hbd| 3-hydroxybutyryl-CoA dehydrogenase	26.34	71.41	42.92	Firmicutes	Clostridiales
ZBCMv2_4940002	ID:5766107	Propanoyl-CoA C-acyltransferase	41.79	52.83	30.26	Euryarchaeota	Archaeoglobales
ZBCMv2_28370001	ID:5771082	putative 6-Oxo-cyclohex-1-ene-carbonyl-CoA hydrolase (Oah, BamA)	38.43	71.86	27.38	Proteobacteria	Rhodocyclales
ZBCMv2_24250001	ID:5770389	putative acetyl-CoA synthetase family protein, ligase (fragment), similar to benzoyl-CoA ligase (BclA, BadA)	20.59	8.22	6.42	Proteobacteria	Rhodobacterales
C1 metabolism, CO2 fixation							
ZBCMv2_5190001	ID:5766178	acsC| Acetyl-CoA synthase corrinoid Fe-S protein, large subunit	39.45	82.40	50.67	Firmicutes	Clostridiales
ZBCMv2_5190002	ID:5766179	Carbon monoxide dehydrogenase/acetyl-CoA synthase subunit alpha (fragment)	43.56	36.52	22.7	Firmicutes	Clostridiales
ZBCMv2_7850002	ID:5766945	Carbon monoxide dehydrogenase/acetyl-CoA synthase subunit alpha (fragment)	15.44	2.75	7.46	Firmicutes	Clostridiales
ZBCMv2_12070002	ID:5767976	Carbon monoxide dehydrogenase/acetyl-CoA synthase subunit beta (fragment)	47.93	8.50	6.71	Firmicutes	Clostridiales
ZBCMv2_7850001	ID:5766944	Carbon monoxide dehydrogenase/acetyl-CoA synthase subunit beta	59.71	9.35	7.25	Firmicutes	Thermoanaerobacterales
ZBCMv2_12070001	ID:5767975	Carbon monoxide dehydrogenase/acetyl-CoA synthetase subunit alpha (fragment)	15.23	16.75	50.38	Firmicutes	Clostridiales
ZBCMv2_38200001	ID:5772649	folD| Bifunctional methylenetetrahydrofolate dehydrogenase / methenyltetrahydrofolate cyclohydrolase	29.16	15.65	17.22	Firmicutes	Clostridiales

ZBCMv2_8840001	ID:5767201	L-carnitine dehydratase/bile acid-inducible protein F (fragment 1)	40.67	7.82	10.42	Euryarchaeota	Archaeoglobales
ZBCMv2_8840003	ID:5767203	L-carnitine dehydratase/bile acid-inducible protein F (fragment 3)	18.87	6.40	11.8	Euryarchaeota	Archaeoglobales
ZBCMv2_310001	ID:5764376	metF| Methylenetetrahydrofolate reductase	32.78	57.31	49.49	Firmicutes	Clostridiales
ZBCMv2_16570002	ID:5768928	panB| 3-methyl-2-oxobutanoate hydroxymethyltransferase	29.61	10.17	15.11	Firmicutes	Clostridiales
ZBCMv2_2390001	ID:5765281	porC| pyruvate:ferredoxin oxidoreductase subunit C (fragment)	5.88	10.91	38.18	Proteobacteria	Syntrophobacterales
ZBCMv2_23140001	ID:5770181	Pyruvate-flavodoxin oxidoreductase (fragment)	47.00	7.96	7.19	Firmicutes	Clostridiales
ZBCMv2_24250002	ID:5770390	putative acetyl-CoA synthetase, ligase (fragment)	21.32	34.00	29.73	Euryarchaeota	Halobacteriales
Dissimilatory sulfate reduction							
ZBCMv2_9130001	ID:5767275	aprA| Adenylylsulfate reductase, alpha subunit (fragment)	48.81	268.04	65.21	Firmicutes	Clostridiales
ZBCMv2_14980003	ID:5768615	aprA| Adenylylsulfate reductase, alpha subunit (fragment)	14.73	58.11	46.04	Firmicutes	Clostridiales
ZBCMv2_14980004	ID:5768616	aprA| Adenylylsulfate reductase, alpha subunit (fragment)	5.32	20.72	62	Firmicutes	Clostridiales
ZBCMv2_15420001	ID:5768706	aprA| Adenylylsulfate reductase, alpha subunit (fragment)	14.68	38.02	34.68	uncultured	uncultured
ZBCMv2_15420002	ID:5768707	aprA| Adenylylsulfate reductase, alpha subunit (fragment)	28.12	8.49	8.91	unidentified	unidentified
ZBCMv2_15420003	ID:5768708	aprA| Adenylylsulfate reductase, alpha subunit (fragment)	22.04	25.36	36.18	unclassified	unclassified
ZBCMv2_36490001	ID:5772375	aprA| Adenylylsulfate reductase, alpha subunit (fragment)	35.66	14.33	13.29	Proteobacteria	Syntrophobacterales
ZBCMv2_14980002	ID:5768614	aprB| Adenylylsulfate reductase, beta subunit	16.46	161.78	70.27	Firmicutes	Thermoanaerobacterales
ZBCMv2_8180002	ID:5767031	dsrA| Sulfite reductase, dissimilatory-type subunit alpha (fragment)	23.11	9.55	8.21	uncultured	uncultured
ZBCMv2_31790002	ID:5771646	dsrA| Sulfite reductase, dissimilatory-type subunit alpha (fragment)	8.74	2.23	9.09	unidentified	unidentified
ZBCMv2_31790003	ID:5771647	dsrA| Sulfite reductase, dissimilatory-type subunit alpha (fragment)	3.37	2.58	25.93	uncultured	uncultured
ZBCMv2_5790001	ID:5766357	dsrA| Sulfite reductase, dissimilatory-type subunit alpha	41.08	75.25	46.98	Firmicutes	Clostridiales
ZBCMv2_12930002	ID:5768170	dsrB| Sulfite reductase, dissimilatory-type subunit	35.08	3.40	3.83	Proteobacteria	Syntrophobacterales

		beta (fragment)					
ZBCMv2_12930003	ID:5768171	dsrB| Sulfite reductase, dissimilatory-type subunit beta (fragment)	17.67	2.42	6.21	unidentified	unidentified
ZBCMv2_5790002	ID:5766358	dsrB| Sulfite reductase, dissimilatory-type subunit beta	44.82	127.59	50.25	Firmicutes	Clostridiales
ZBCMv2_29500001	ID:5771276	dsrC| Sulfite reductase, dissimilatory-type subunit gamma	12.08	5.33	20.72	Firmicutes	Clostridiales
ZBCMv2_4810002	ID:5766068	sat| Sulfate adenylyltransferase, ATP sulfurylase (fragment)	31.89	85.98	59.31	Firmicutes	Clostridiales

3.3 Qualitive and quantitive proof of ethylbenzene biodegradation at a multi-contaminatedfield site in Òdena, Spain

To gain an insight into BTEX biodegradation in this multi-contaminated field site, two sampling campaigns were carried out in November, 2014 and November, 2015. Samples for concentration and isotope signature analysis were taken both in 2014 and 2015. Materials for microcosms were taken in 2014. The microcosms were enriched for the study of ethylbenzene degradation under difference redox condition.

3.3.1 Concentration of BTEX compounds in different wells in the contaminated site

The sampling campaigns in 2014 and 2015 took samples mainly from three wells: S3, S6 and S9 (Figure 2.1). The ground water flow direction was from S3 to S6. S9 was located downstream of S3 and S6. The BTEX concentration decreased with the flow direction and in the well S3, the contaminant concentrations were much higher than in the other two. Toluene had the highest concentration of the BTEX contaminants, followed by xylene and ethylbenzene; benzene had the lowest concentration (Tables 3.7, 3.8, 3.9). The measured concentrations in 2014 were lower in comparison to the other two years. This was probably due to the difference in the used analytical methods. In 2014, the samples were firstly extracted with pentane and measured with GC-MS. The efficiency of extraction would probably directly affect the measured concentration. Significant seasonal variation was also shown in the previous studies of this contaminated site (Palau *et al.* 2014) . In comparison, in the years 2013 and 2015, the samples were measured directly by headspace analysis using a GC-MS. The concentration of toluene and ethylbenzene decreased slightly in the year 2015 when compared to the year 2013.

Table 3.7 BTEX contaminants concentration at different depth in March 2013 at the field site of Òdena, Spain

2013	Benzene (µg/L)	Toluene (µg/L)	Ethylbenzene (µg/L)
S3-11.5*	11.5	1110.3	298.8
S3-13.5	11.8	947.7	337.1
S3-15.5	10.8	1510.2	367.4
S3-17.5	50.3	3535.2	243.4
S3-19.5	55.6	2428.1	107.4
S6-9.5	2.7	n.d.	14.6
S6-11	n.d.**	3.72	16.8
S6-12.5	5.8	n.d.	14.7
S6-14.5	n.d.	n.d.	14.7

S6-17.5	n.d.	n.d.	14.9
S9-9	n.d.	n.d.	14.6
S9-11	n.d.	n.d.	14.6
S9-13	n.d.	n.d.	14.6
S9-15	n.d.	n.d.	14.6
S9-17	n.d.	n.d.	14.5
S9-19	n.d.	n.d.	14.6

(Data provided by Dr. Rosell in the working group of Prof.Dr.Albert Soler, Department of Mineralogy, Petrology and Applied Geology, University Barcelona)

* S3 - 11.5: it means 11.5 m below the ground surface. The same goes for other sampling point.

**: n.d.: not determined

Table 3.8 BTEX contaminants concentration at different depth in November 2014 at the field site of Òdena, Spain

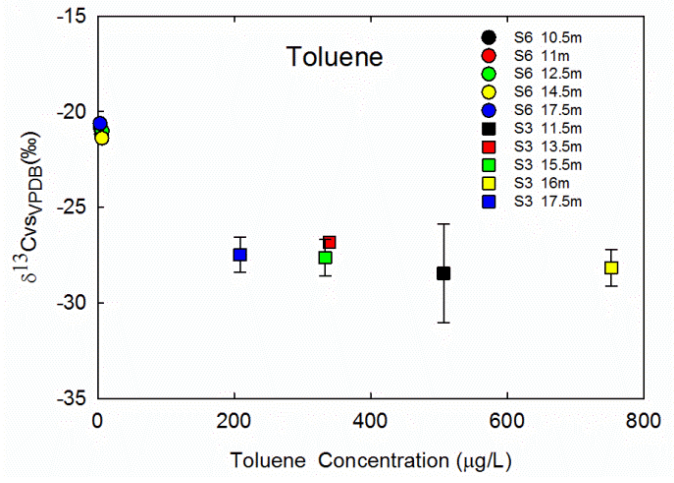
2014	Benzene (µg/L)	Toluene (µg/L)	Ethylbenzene (µg/L)	*m,p*-Xylene (µg/L)	*o*-Xylene (µg/L)
S3-11.5	n.d.	506.6	57.4	246.7	n.d.
S3-13.5	n.d.	413.8	56.3	123.3	5.1
S3-15.5	n.d.	333.0	40.4	123.3	2.9
S3-16	n.d.	752.4	41.3	123.3	6.1
S3-19.5	n.d.	208.3	67.7	123.3	n.d.
S6-Overall	n.d.	1.95	11.6	52.9	2
S9-Overall	n.d.	n.d.	n.d.	52.9	n.d.

Table 3.9 BTEX contaminants concentration at different depth in November 2015 at the field site of Òdena, Spain

2015	Benzene (µg/L)	Toluene (µg/L)	Ethylbenzene (µg/L)	*m,p*-Xylene (µg/L)	*o*-Xylene (µg/L)
S3-Overall	36.5	3587.8	483.8	828.6	76.9
S3-13.5	23.0	1500.6	171.2	300.7	32.7
S3-17.5	21.3	939.4	72.0	111.2	14.3
S6-Overall	6.1	6.5	12.1	1.1	0.8
S6-10.5	5.7	4.1	6.4	0.8	0.6
S6-11	5.5	5.4	10.5	n.d.	0.7
S6-12.5	5.9	6.3	10.9	1.0	0.7
S6-14.5	5.6	5.9	10.2	1.0	0.7
S6-17.5	4.9	3.3	4.3	0.7	0.6
S9-11	0.8	0.1	0.1	n.d.	n.d.
S9-13	0.8	n.d.	0.1	n.d.	n.d.
S9-15	0.8	n.d.	0.2	0.1	n.d.

3.3.2 Carbon and hydrogen stable isotope analysis of BTEX compounds in different wells of the contaminated site

Due to the low concentration of benzene in the sampling wells, benzene was not analyzed for the stable isotopes. Within each individual well, the values of $\delta^{13}C$ and $\delta^{2}H$ for toluene, ethylbenzene and *m,p*-xylene did not present significant changes at different depth level (Figures 3.29 and 3.30). An obvious decrease in BTEX concentrations between S3 and S6 in the flow direction was observed. According to the concentration data in the year of 2015, a decrease of concentration from 3587.8 µg/L to 6.5 µg/L, from 483.8 µg/L to 12.1 µg/L, from 828.6 µg/L to 1.1 µg/L and from 76.9 µg/L to 0.8 µg/L was observed accordingly for toluene, ethylbenzene, *m,p*-xylene and *o*-xylene from well S3 to S6 in the groundwater flow direction. In comparison, an increase in $\delta^{13}C$ values in the flow direction between S3 and S6 was obtained: the $\delta^{13}C$ value of toluene increased from -27.7 ‰ ± 0.6 ‰ to -21.0 ‰ ± 0.3‰; the $\delta^{13}C$ value of ethylbenzene increased from -25.4 ‰ ± 0.5‰ to -22.0 ‰ ± 1.2‰ and the $\delta^{13}C$ value of *m,p*-xylene increased from -28.6 ‰ ± 0.8 ‰ to -18.9 ‰ ± 1.5‰ (Figure 3.29-3.30). Since the method detection limits of hydrogen stable isotope analysis are much higher than carbon stable isotope, only samples from well S3 could be analyzed for the hydrogen stable isotopes. The two dimensional plot (carbon and hydrogen stable isotope values) of samples from well S3 are shown in figure 3.31. Minor changes were observed for carbon and hydrogen isotope values with the depth which is discussed in details in chapter 4.4.1. The isotopic values of carbon and hydrogen for samples from well S9 were not analyzed because of the low BTEX concentrations which could not reach the detection limit of the used analytical methods. The decrease in concentration and increase in $\delta^{13}C$ values of BTEX compounds indicated a degradation process in the groundwater flow direction from well S3 to S6.



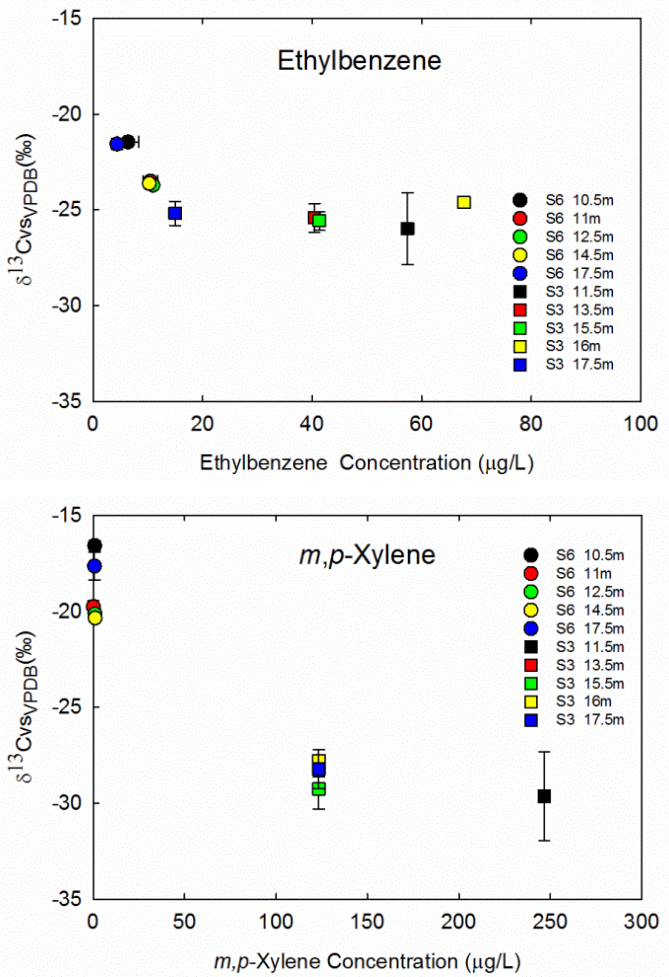
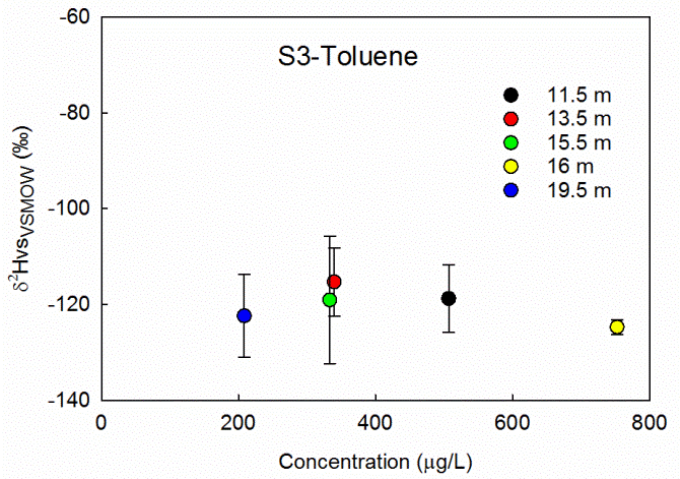


Figure 3.29 $\delta^{13}C$- concentration plots of toluene, ethylbenzene and *m,p*-xylene at different levels of wells S3 and S6.



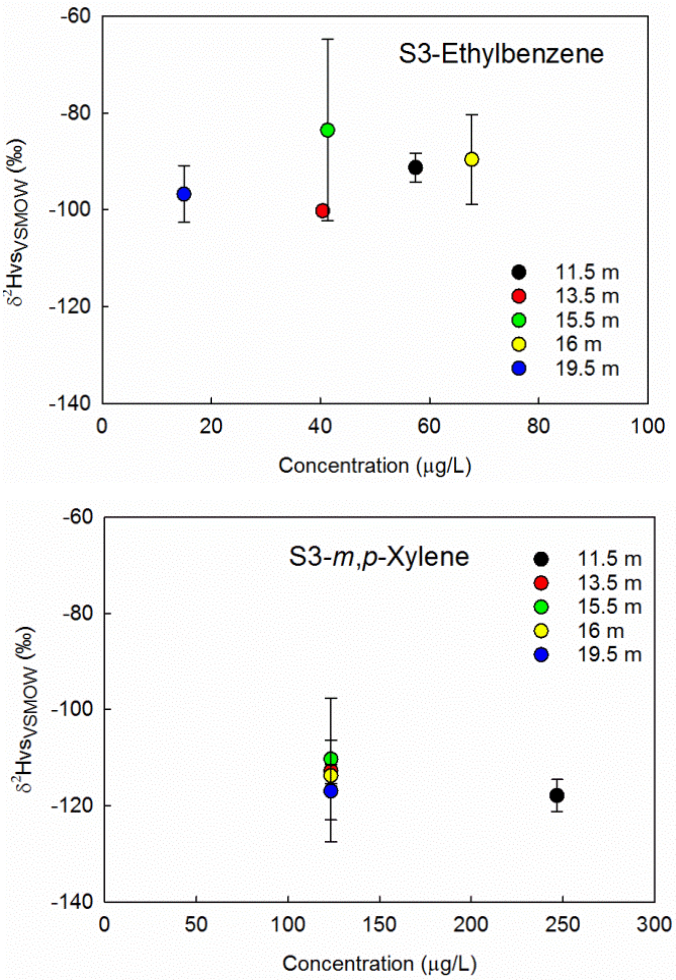
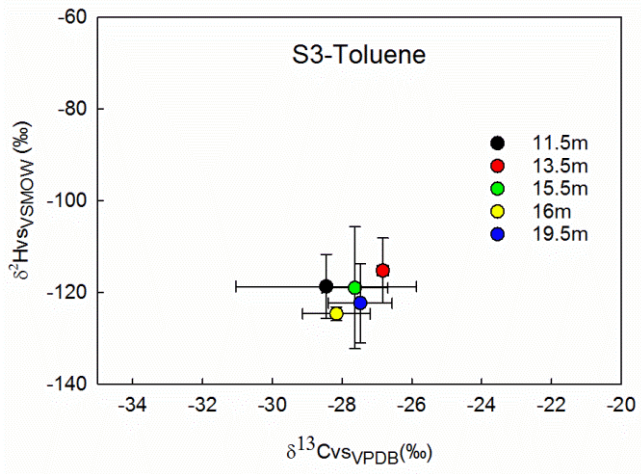
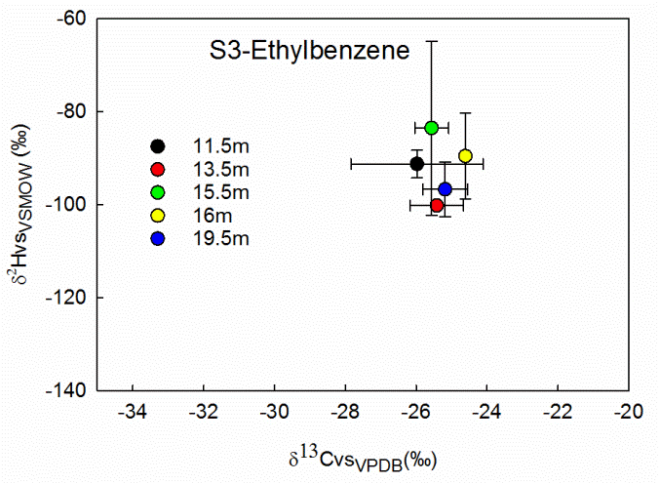


Figure 3.30 δ^2H- concentration plots of toluene, ethylbenzene and *m,p*-xylene at different levels of well S3. Due to the relatively low concentration of BTEX in well S6, detection of δ^2H signatures was not possible.





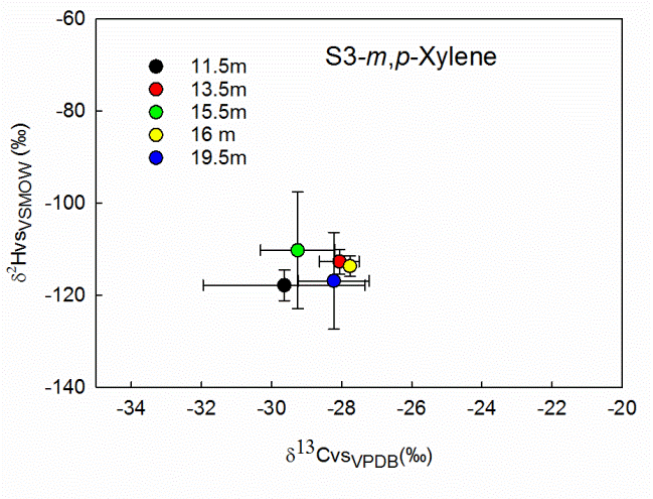
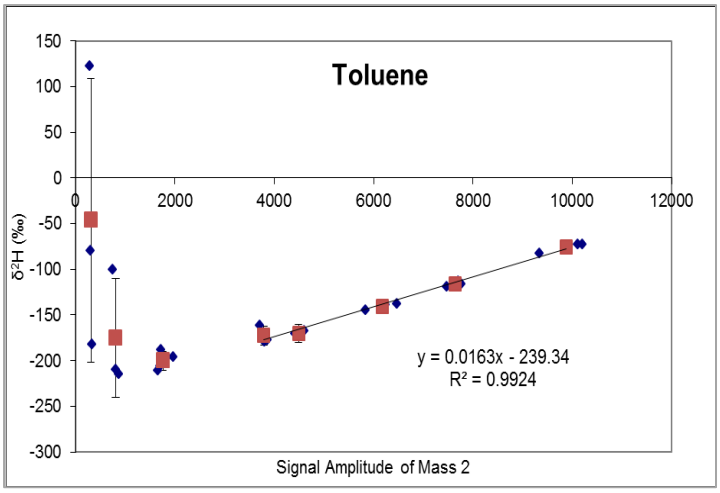
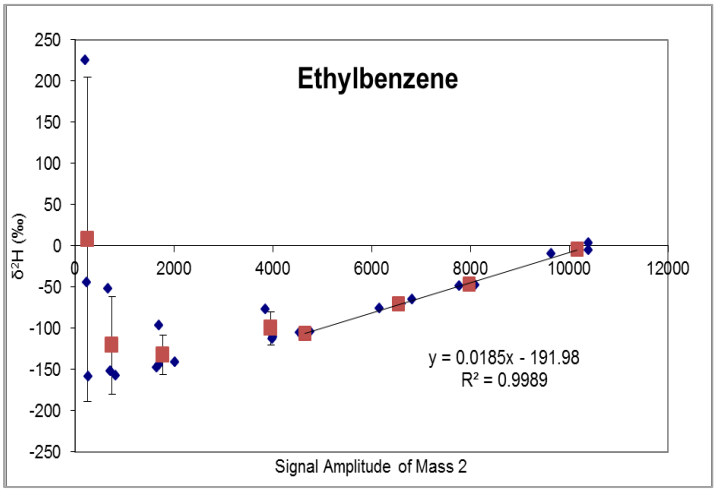


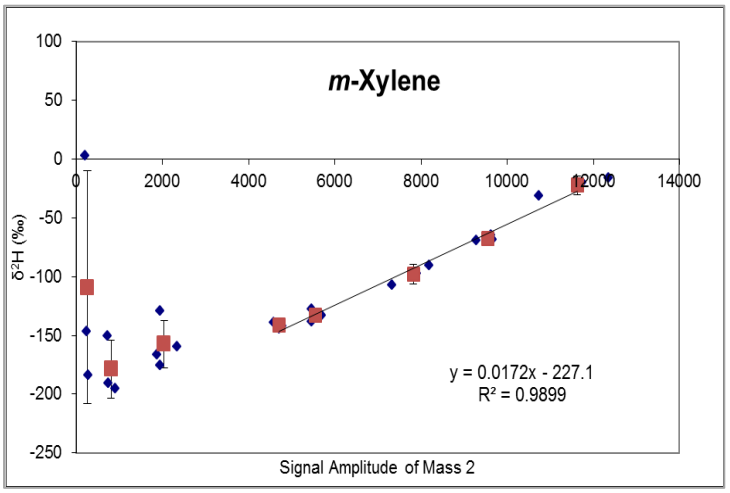
Figure 3.31 $\delta^{13}C$- $\delta^{2}H$ plots of toluene, ethylbenzene and *m,p*-xylene.

3.3.3 Method development and validation of BTEX hydrogen isotope analysis at low concentration by large volume injection

The low concentration of organic compounds in the environmental samples is always a challenge in method development for stable isotope analysis. In order to analyze the hydrogen isotopic signature in samples with low concentrations from S6 well, the HS-PTV-GC/IRMS method described by Dr. Herrero-Martín (Herrero-Martin *et al.* 2015) was used with modification in this study. BTEX lab standards were analyzed prior to the samples. However, the $\delta^{2}H$ values changed with concentration (Figure 3.32 and 3.33) and with the insertion of backflush in the method (Figure 3.33), the $\delta^{2}H$ values showed also changes. Therefore, the method development with large volume injection method was not successful.


Toluene
150
100
50
0
-50
-100
-150
-200
-250
-300
0
2000
4000
6000
8000
10000
12000
δ²H (‰)
y = 0.0163x - 239.34
R² = 0.9924
Signal Amplitude of Mass 2


Ethylbenzene
250
200
150
100
50
0
-50
-100
-150
-200
-250
0
2000
4000
6000
8000
10000
12000
δ²H (‰)
y = 0.0185x - 191.98
R² = 0.9989
Signal Amplitude of Mass 2


m-Xylene
100
50
0
-50
-100
-150
-200
-250
0
2000
4000
6000
8000
10000
12000
14000
δ²H (‰)
y = 0.0172x - 227.1
R² = 0.9899
Signal Amplitude of Mass 2

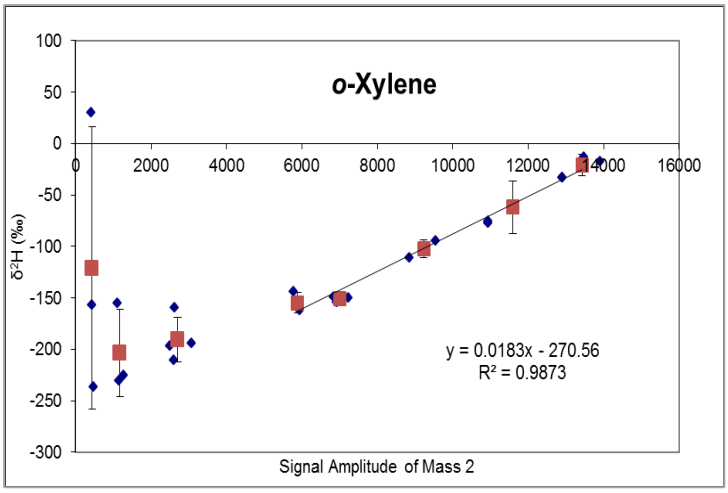
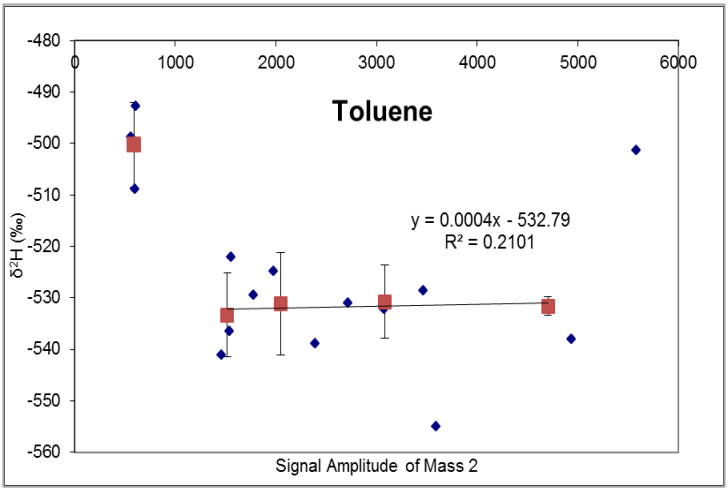
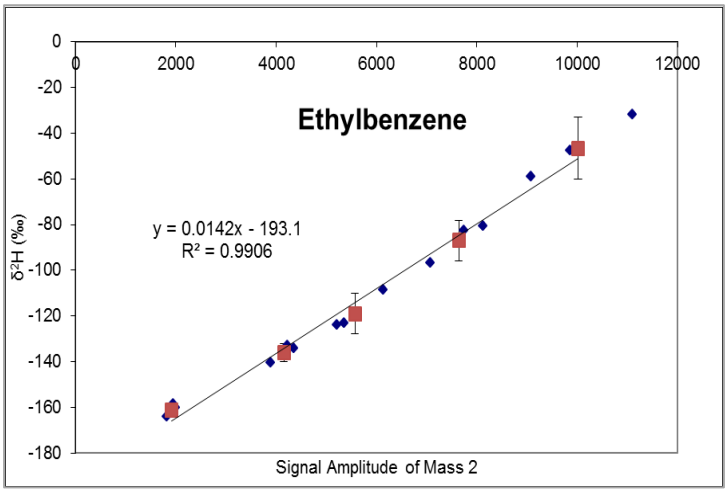


Figure 3.32 Representative calibration lines of BTEX standards using the HS-PTV-GC/IRMS method. The blue diamonds relate to δ^2H values. The red squares represent the δ^2H mean value of samples at the same concentration. No backflush was inserted among the four peaks of the analyzed compounds.





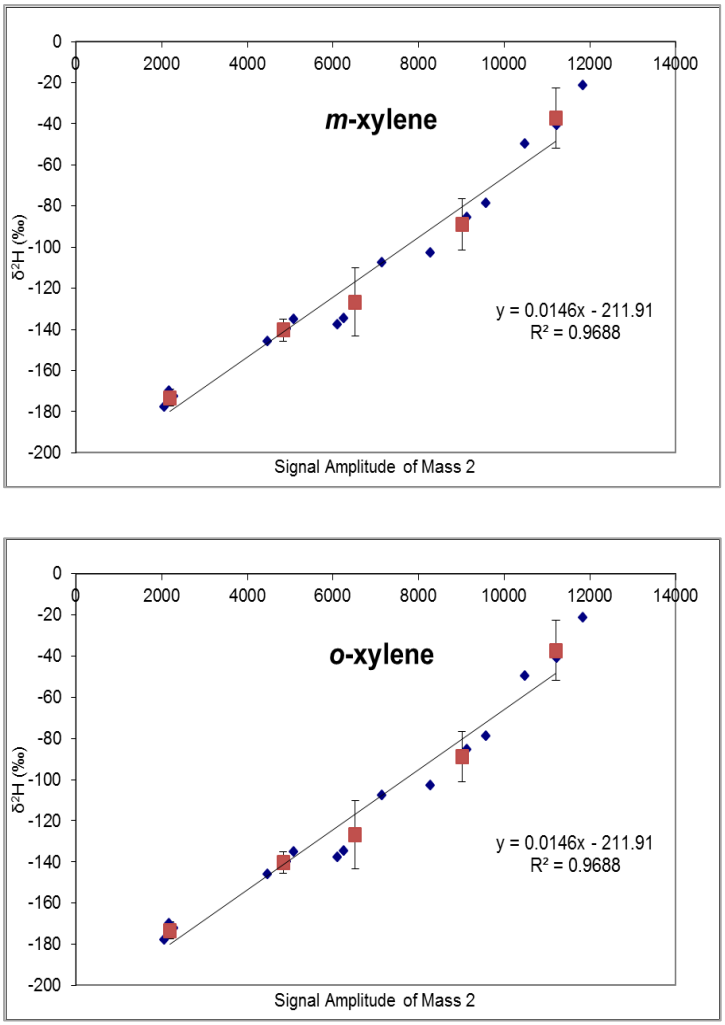


Figure 3.33 Representative calibration lines of BTEX standards using the HS-PTV-GC/IRMS method. The blue diamonds relate to δ^2H values. The red squares represent the δ^2H mean value of samples at the same concentration. Backflush was inserted among the four peaks of the analyzed compounds.

3.3.4 Growth kinetics of ethylbenzene-degrading enrichment cultures using sludge materials from different wells

The redox potential of the three sampling wells was monitored by the cooperation working group of the University Barcelona. Redox gradients were detected in different wells along the groundwater flow. The redox conditions in the field site were determined by analyzing the concentrations of redox sensitive compounds (dissolved oxygen concentration (DOC), NO_3^-/NO_2^-, SO_4^2 and dissolved Mn, Fe). The redox condition is assigned according to the criteria of McMahon and Capelle, which identifies the redox processes in aquifer systems by defining the threshold concentrations of dissolved oxygen, NO_3^-, $Mn^{2+,}$ Fe^{2+} and SO_4^{2-} of each redox process(McMahon and Chapelle 2008). For

example, the nitrate-reducing condition is indicated by: concentration of dissolved oxygen< 0.5 mg/L, $NO_3^- \geqslant 0.5$ mg/L, Mn^{2+}<0.05 mg/L, Fe^{2+}<0.1 mg/L and the Fe(III)/sulfate-reducing condition is indicated by concentration of dissolved oxygen< 0.5 mg/L, NO_3^-<0.5 mg/L, $Fe^{2+} \geqslant 0.1$ mg/L and $SO_4^{2-} \geqslant 0.1$ mg/L (McMahon and Chapelle 2008). The bottom of well S3 was determined as sulfate or iron reducing conditions. Decrease in sulfate concentration with the increase of depth and an enrichment of $\delta^{34}S$ in SO_4^{2-} might indicate the use of sulfate as terminal electron acceptor. The well S6, was analyzed as sulfate or iron or nitrate reducing condition and the well S9 was regarded as oxic or nitrate reducing condition (unpublished results, personal communication with Dr. Rosell in the working group of Prof. Dr. Albert Soler, Department of Mineralogy, Petrology and Applied Geology, University Barcelona). A schematic picture of the modelled redox conditions of the sampling wells is shown in figure 3.34.

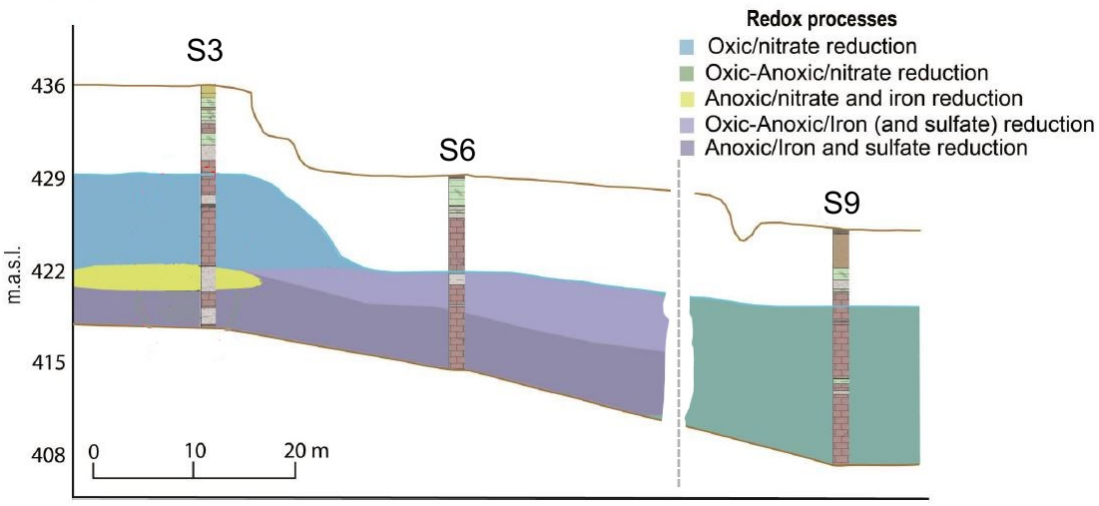
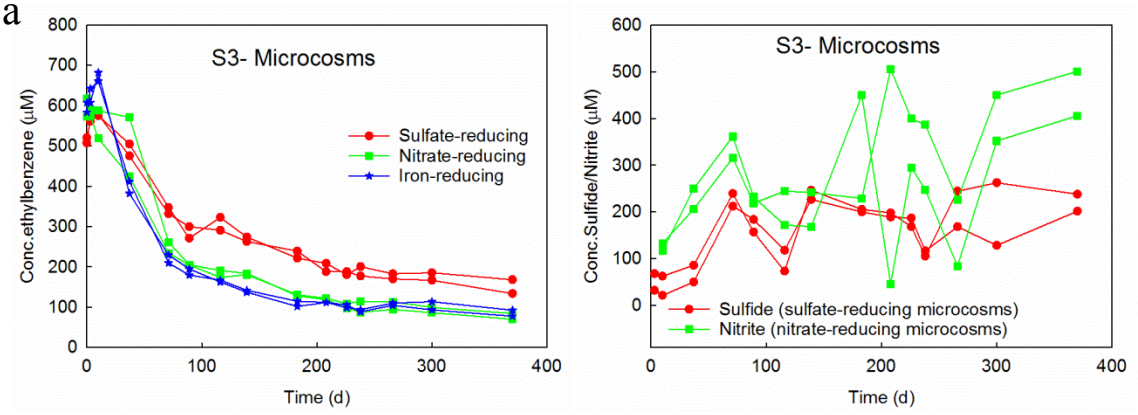


Figure 3.34 Modelled redox conditions of a transect in the sampling wells at the contaminated site Òdena, Spain. The depth is expressed in meters above sea level (m.a.s.l.).
(Unpublished results, personal communication with Dr. Rosell in the working group of Prof. Dr. Albert Soler, Department of Mineralogy, Petrology and Applied Geology, University Barcelona).

Microcosms were set up using sludge sampled from the bottom of the three wells. Ethylbenzene was chosen as model substrate for microbial cultures as it was a main hydrocarbon contaminant at the site and degradation pattern under anoxic conditions are still not well understood. For the microcosms which were set up with sludge from well S3, there was a decrease of around 400 µM in ethylbenzene concentration in all cultures in around 380 days of cultivation (Figure 3.35a). This is however no convincible evidence of microbial degradation as there is almost the same amount of substrate loss in the

abiotic control (Figure 3.35d). No obvious acceleration in the substrate loss rate was observed during the cultivation time (Figure 3.35a). There is an increase of sulfide concentration by around 150 µM in cultures grown under sulfate-reducing condition and an increase of nitrite concentration by approximately 400 µM in cultures grown under nitrate-reducing condition. Color change from thin reddish brown to grey-green was observed for the cultures grown under iron-reducing condition. This indicated the reduction of Fe(III) to Fe(II). There is in comparison no significant accumulation of sulfide or nitrite in the substrate-free control. For the microcosms which were set up with sludge from well S6, ethylbenzene was biodegraded under nitrate-reducing conditions. An increase of nitrite concentration by around 600 µM was observed. An acceleration of degradation started at around 100 days of the cultivation and it kept increasing after twice re-spike of substrate (Figure 3.35b). Ethylbenzene biodegradation cannot be proved under the other two conditions. The substrate-loss rate was similar to that in the abiotic control. No obvious accumulation of sulfide was observed under sulfate-reducing condition.There is a color change from thin reddish brown to grey-green of the cultures set up under iron-reducing condition which indicated the production of ferrous iron. For the microcosms which were set up with sludge from well S9, the situation was similar to those for S6 and an acceleration of degradation started at around 200 days for cultures under nitrate-reducing condition (Figure 3.35c). Two bottles of microcosms were set up for every single condition. The duplicate microcosms behaved in general similarly to each other during the cultivation. Moreover, microcosms were set up with sludge from S9 under aerobic conditions using toluene as growth substrate as well. The microcosms showed fast degradation ability of toluene (data not shown).

a



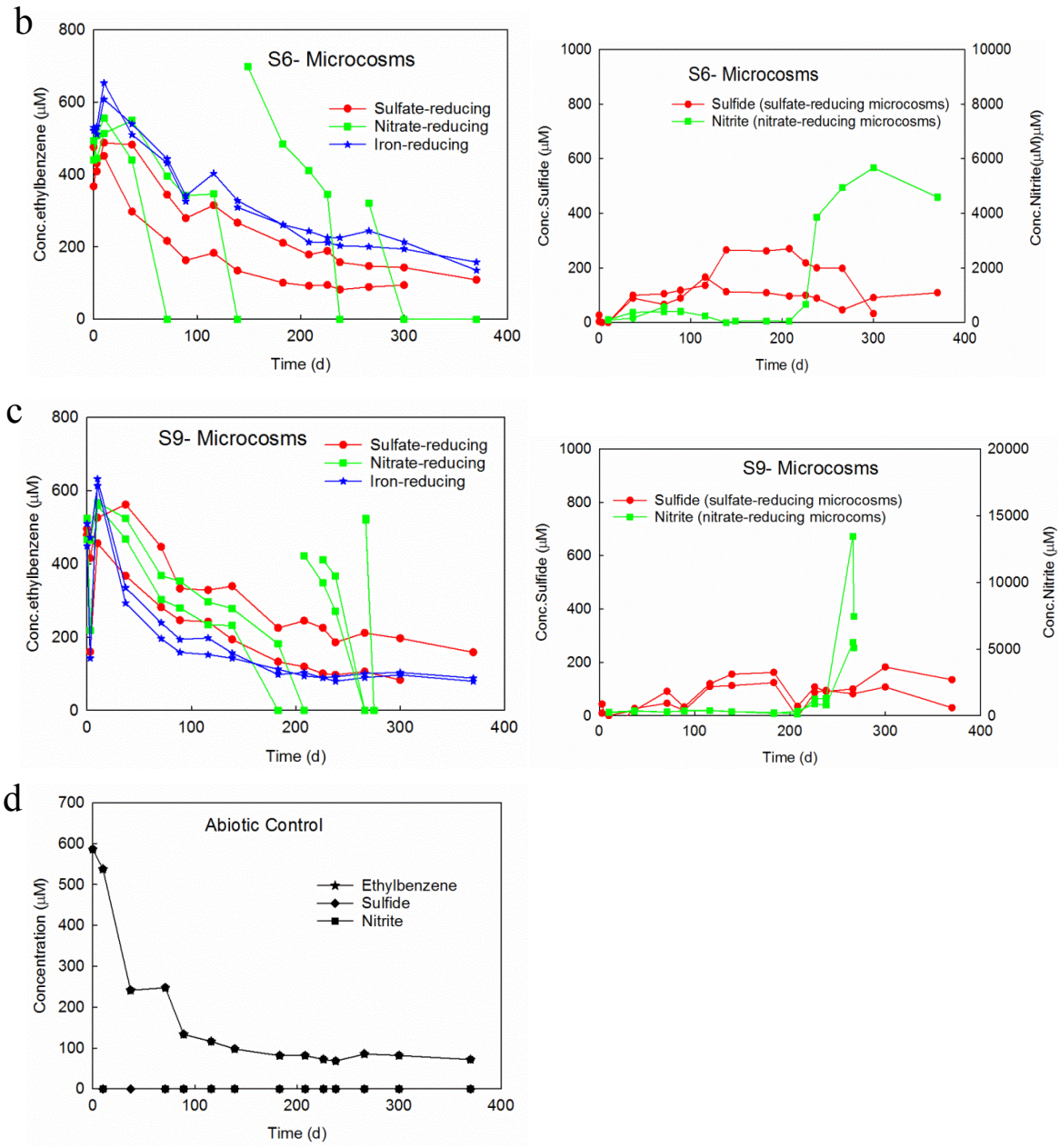


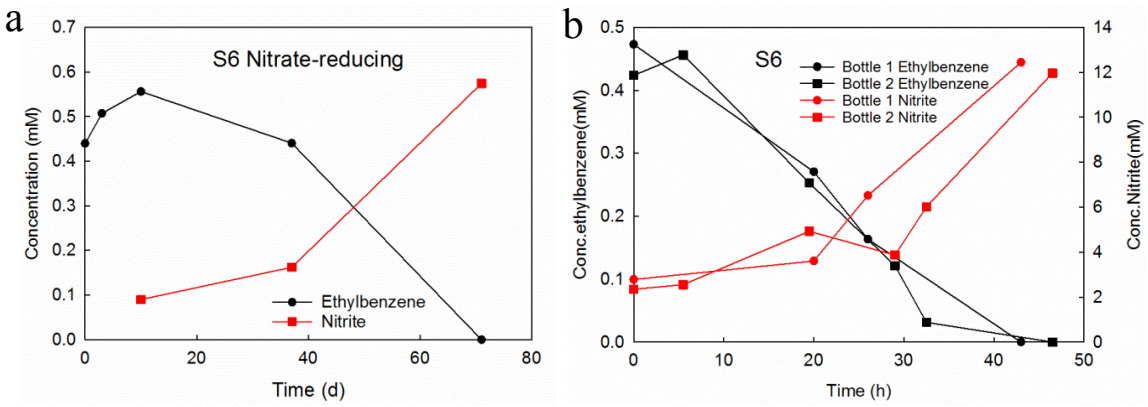
Figure 3.35 Degradation kinetics of microcosms set up with sludge from sampling wells of the Òdena site . The curves in red represent the microcosms set up under sulfate-reducing condition; in blue represent those under iron-reducing condition; in green nitrate-reducing condition and in black the abiotic control. a) Microcosms set up with sludge from well S3; b) microcosms set up with sludge from well S6; c) microcosms set up with sludge from well S9; d) the abiotic control.

3.3.5 Carbon stable isotope fractionation analysis of two ethylbenzene-degrading cultures

The microcosms which were set up with sludge from S6 and S9 under nitrate-reducing condition were then transferred and inoculated into fresh medium for further enrichment.

After four sub-cultivation steps, the degradation rate of ethylbenzene under nitrate reducing condition increased from 4.86 µM/day to 264 µM/day for the S6 microcosms and from 2.56 µM/day to 264 µM/day for the S9 microcosms (Figure 3.36).

The fifth sub-cultures of both S6 and S9 microcosms were used to perform ethylbenzene isotope fractionation experiments (degradation kinetics shown in figure 3.36). In case of the S6 microcosm, approximately 448.5 µM ethylbenzene was degraded within 45 hours with a production of around 9 mM nitrite (Figure 3.36). At the same time, the $\delta^{13}C$ values increased from -30.5 ‰ to -20.2 ‰. A bulk enrichment factor of -3.9 ± 0.4 was determined accordingly (Figure 3.37). For the S9 microcosm, the degradation of 518 µM ethylbenzene took around 51 hours accompanied by a production of 4.9 mM nitrite (Figure 3.36). An enrichment of $\delta^{13}C$ values from -30.2 ‰ to -18.2 ‰ was observed and a corresponding bulk enrichment factor of -3.7 ± 0.4 was calculated (Figure 3.37). The AKIEc values for S6 and S9 were 1.0322 ± 0.0034 and 1.0305 ± 0.0034, accordingly (Table 3.10). The bulk enrichment factors and $AKIE_c$ values are in accordance to those for *Aromatoleum aromaticum* EbN1 ($\varepsilon_{bulk,c}$ -3.8 ± 0.1‰, AKIEc 1.03 ± 0.001) and *Georgfuchsia toluolica* G5G6 ($\varepsilon_{bulk,c}$ -4.1 ± 0.2‰, AKIEc 1.033 ± 0.001) using side-chain mobohydroxylation by ethylbenzene dehydrogenase as initial, isotope-fractionating step of the ethylbenzene degradation pathwaywhich side-chain monohydroxylation was the initial step in the degradation pathway (Dorer *et al.* 2014).



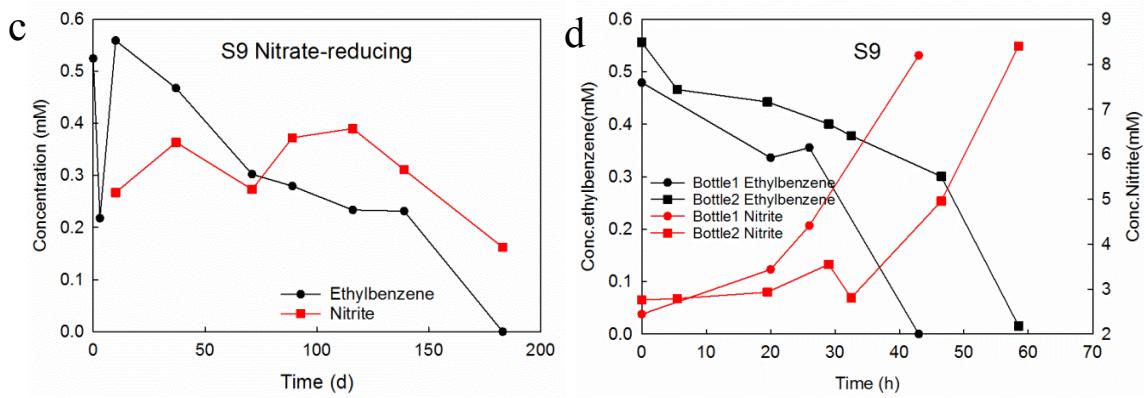


Figure 3.36 Growth kinetics of microcosms set up with sludge from sampling wells S6 and S9 under nitrate reducing condition. The curves in black represent ethylbenzene concentration and in red represent the nitrite concentration. a) original microcosms set up with sludge from well S6; b) the fifth sub-cultivation cultures of S6; c) original microcosms set up with sludge from well S9; d) the fifth sub-cultivation cultures of S9.

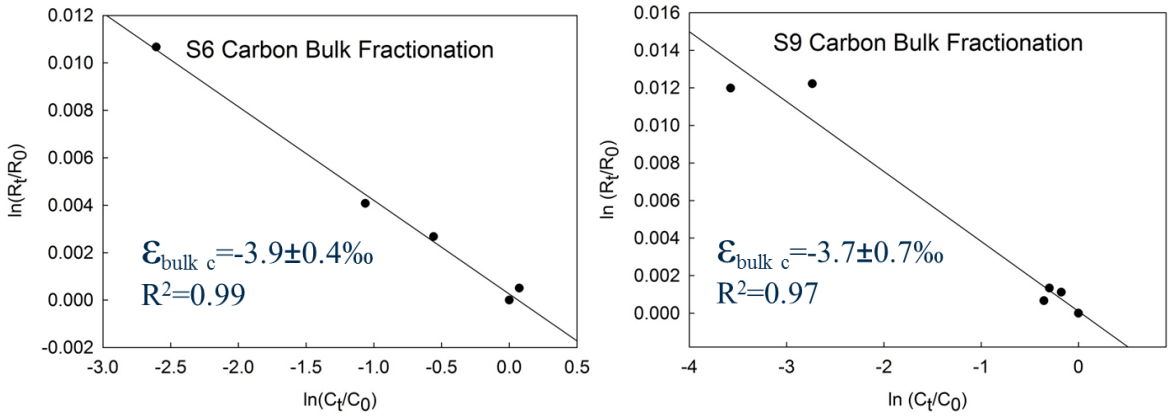


Figure 3.37 Rayleigh plots of carbon isotope fractionation during ethylbenzene degradation of the enrichment cultures S6 and S9. Bulk enrichment factors were calculated from the slope of the regression curve and linearity is expressed in r^2 values.

Table 3.10 Bulk enrichment factors and AKIEc values upon ethylbenzene degradation by enrichment cultures (The equations are listed in chapter 4.7).

Microcosm	Substrate and terminal electron acceptor	Possible initial step of the degradation pathway)	$\varepsilon_{bulk,c}$ (‰)	$AKIE_c$
S6	ethylbenzene, nitrate	Side-chain monohydroxylation	-3.9 ± 0.4	1.0322 ± 0.0034
S9	ethylbenzene, nitrate	Side-chain monohydroxylation	-3.7 ± 0.4	1.0305 ± 0.0034

3.3.6 16S rRNA sequencing results of the two ethylbenzene-degrading cultures

The enrichment cultures S6 and S9 were transferred to fresh medium (20% [v/v] inoculum) for several times to obtain well-grown cultures for the sequencing. Genomic DNA was extracted from the enrichment culture and the bacterial 16S rRNA gene fragments were amplified and sequenced. For both cultures, only a single sequence was detected which indicated the S6 and S9 cultures were almost pure or pure. The sequence results are shown in Table 3.11.

Table 3.11 The 16s rRNA gene sequence from enrichment cultures S6 and S9

Culture	Sequence
S6	TCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGC GGGGGCTTCGGCCTGCCGGCGAGTGGCGAACGGGTGAGTAATGCATCGGA ACGTACCCAGTCGTGGGGGATAACTACGCGAAAGCGTAGCTAATACCGCA TACGCCCTGAGGGGGAAAGCGGGGGACCTTCGGGCCTCGCGCGATTGGAG CGGCTGATGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGA CGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACA CGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGC GCAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGT AAAGCTCTTTCAGACGGAAAGAAATCGGGCGCGTGAATAGCGTGCCTGGA TGACGGTACTGTCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCG GTAATACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTG CGCAGGCGGTTGTGTAAGACAGGTGTGAAATCCCCGGGCTTAACCTGGGA ACTGCGCTTGTGACTGCACGGCTAGAGTACGGCAGAGGGGGGTGGAATTC CACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAACACCGATGGCGAA GGCAGCCCCCTGGGCCGATACTGACGCTCATGCACGAAAGCGTGGGGAGC AAACAGG
S9	ATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCGGGG GCTTCGGCCTGCCGGCGAGTGGCGAACGGGTGAGTAATGCATCGGAACGT GCCCATGTCGTGGGGGATAACGTATCGAAAGGTACGCTAATACCGCATAC GTCCTGAGGGAGAAAGCGGGGGATTCTTCGGAACCTCGCGCGATTGGAGC GGCCGATGTCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGAC GATCCGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTGAGACAC GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGG

	CAACCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTA AAGCTCTTTCGGCCGGGAAGAAATCGCGCACTCTAACATAGTGTGTGGAT GACGGTACCGGACTAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGG TAATACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGC GCAGGCGGTTTTGTAAGACAGATGTGAAATCCCCGGGCTCAACCTGGGAA CTGCGTTTGTGACTGCAAGGCTAGAGTACGGCAGAGGGGGGTGGAATTCC TGGTGTAGCAGTGAAATGCGTAGATATCAGGAGGAACACCGATGGCGAAG GCAGCCCCCTGGGCCTGTACTGACGCTCATGCACGAAAGCGTGGGGAGCA AACAGGATTAGATACCCTGGTAGTCCAC

The sequences were blasted on basis of the RDP sequence match tool. The S6 sequence was assigned to the genus *Azoarcus* (Betaproteobacteria, the order Rhodocyclales). It had the most similarity with the strain *Azoarcus sp*. PbN1 (similarity score of 1). The S9 sequence was assigned to the genus *Thauera* (Betaproteobacteria, the order Rhodocyclales). It shared the highest similarity with the strain *Thauera chlorobenzoica* (similarity score of 0.991).The complete list of match result is shown in figure below.

rootrank Root (0/20/1558793) (selected/match/total RDP sequences)
domain Bacteria (0/20/1502575)
phylum "Proteobacteria" (0/20/437996)
class Betaproteobacteria (0/20/92636)
order Rhodocyclales (0/20/4822)
family Rhodocyclaceae (0/20/4822)
genus Azoarcus (0/20/271)

S000000311	not_calculated	0.953	1419	Azoarcus anaerobius (T); LuFres1; DSM 12081; Y14701
S000007007	not_calculated	1.000	1404	Azoarcus sp. PbN1; X83532
S000008376	not_calculated	0.982	1406	Azoarcus buckelii (T); type strain: U120 = DSM 14744; AJ315676
S000008918	not_calculated	0.949	1402	Azoarcus sp. EbN1; X83531
S000441868	not_calculated	0.970	1396	uncultured bacterium; PL-33B3; AY570565
S000441922	not_calculated	0.970	1398	uncultured bacterium; PL-33B11; AY570619
S000441923	not_calculated	0.970	1398	uncultured bacterium; PL-33B2; AY570620
S000528908	not_calculated	0.949	1412	Aromatoleum aromaticum EbN1; CR555306
S000528909	not_calculated	0.949	1412	Aromatoleum aromaticum EbN1; CR555306
S000544507	not_calculated	0.986	1357	uncultured bacterium; 5RHF43; AJ863386
S000650143	not_calculated	1.000	1389	uncultured soil bacterium; F45_Pitesti; DQ378207
S000987995	not_calculated	0.983	1393	uncultured Rhodocyclaceae bacterium; D25_12; EU266891
S001036029	not_calculated	0.956	1401	uncultured bacterium; ctg1_topo4_1; EU708500
S001036034	not_calculated	0.956	1402	uncultured bacterium; ctg1_TOPO1-7; EU708505
S001800465	not_calculated	0.974	1388	uncultured bacterium; 4y-91; FJ444757
S001913990	not_calculated	0.970	1292	uncultured bacterium; LL143-2J8; FJ670829
S002480677	not_calculated	0.963	1399	uncultured bacterium; BN109; HQ190355
S002480737	not_calculated	0.963	1399	uncultured bacterium; BR53; HQ190415
S002887206	not_calculated	0.963	1397	uncultured Azoarcus sp.; EK_CK557; JN038235
S003552258	not_calculated	0.965	1402	uncultured bacterium; SN114; JQ825002

Figure 3.38: Sequencing results of culture S6 annotated by SeqMatch tool of RDP.

rootrank Root (0/20/1558793) (selected/match/total RDP sequences)
domain Bacteria (0/20/1502575)
phylum "Proteobacteria" (0/20/437996)
class Betaproteobacteria (0/20/92636)
order Rhodocyclales (0/20/4822)
family Rhodocyclaceae (0/20/4822)
genus Thauera (0/20/477)

☐	S000004968	not_calculated	0.956	1412	Thauera aromatica; S100; AJ315681
☐	S000009314	not_calculated	0.953	1395	Thauera mechernichensis (T); TL1; Y17590
☐	S000387203	not_calculated	0.991	1384	Thauera chlorobenzoica (T); 3CB-1; AF123264
☐	S000389670	not_calculated	0.952	1440	Thauera aromatica; 3CB2; AF229881
☐	S000441926	not_calculated	0.991	1409	uncultured bacterium; PL-34B4; AY570623
☐	S000467742	not_calculated	0.957	1395	Thauera sp. 27; AY838760
☐	S000768429	not_calculated	0.976	1391	Thauera sp. R5; AB287434
☐	S002110041	not_calculated	0.953	1284	uncultured bacterium; ncd844d09c1; HM305015
☐	S002420285	not_calculated	0.953	1411	uncultured Thauera sp.; S-123; HQ132455
☐	S002902715	not_calculated	0.956	1415	uncultured bacterium; Q7450-ASSA; JN391578
☐	S003989188	not_calculated	0.956	1414	uncultured bacterium; s23; KC907102
☐	S004200490	not_calculated	0.956	1405	uncultured beta proteobacterium; 1_38_14|10|Lib1; KJ650697
☐	S004519195	not_calculated	0.953	1315	uncultured bacterium; ALK-1 2-1; KP136297
☐	S004541245	not_calculated	0.953	1315	uncultured bacterium; 55-1; KP641098
☐	S004541251	not_calculated	0.953	1315	uncultured bacterium; 45-1; KP641104
☐	S004541252	not_calculated	0.953	1315	uncultured bacterium; 45-3; KP641105
☐	S004541259	not_calculated	0.953	1315	uncultured bacterium; 35-1; KP641112
☐	S004541262	not_calculated	0.953	1315	uncultured bacterium; 35-4; KP641115
☐	S004541264	not_calculated	0.953	1315	uncultured bacterium; 35-6; KP641117
☐	S004585181	not_calculated	0.957	1395	uncultured Thauera sp.; MBR Ca 300ppm 22; KT182575

Figure 3.39: Sequencing results of culture S9 annotated by SeqMatch tool of RDP.

4 DISCUSSION

4.1 Method development and the identification of biodegradation pathways of phenol and cresols by compound specific isotope analysis

4.1.1 Isotope fractionation of ring monooxygenation

The oxygen-dependent hydroxylation (monooxygenation) of phenol and cresols is catalyzed by monooxygenases, introducing one oxygen atom from molecular oxygen at the aromatic ring or at the methyl moiety (Corvini et al. 2006, Pérez-Pantoja *et al.* 2010). Phenol and *p*-cresol degradation in *A.calcoaceticus* is catalyzed by a phenol hydroxylase acting at the aromatic ring (Paller *et al.* 1995). The enzyme shares high similarity with the phenol hydroxylase of *Pseudomonas sp*. CF600, which belongs to the soluble diiron monooxygenases family and is composed of a reductase subunit, an oxygenase subunit, an activator and a subunit essential for phenol uptake (Ehrt *et al.* 1995, Leahy et al. 2003, Pérez-Pantoja *et al.* 2010). During the phenol or cresol hydroxylation reaction, electrons from NADH are transferred via the FAD and iron-sulfur containing reductase to the binuclear non-heme iron oxygenase. This is followed by the incorporation of one oxygen atom to the aromatic substrate, the other is released in form of H_2O (Powlowski and Shingler 1990, Powlowski and Shingler 1994, Powlowski et al. 1997). The $AKIE_c$ values

obtained upon phenol and *p*-cresol degradation by *A.calcoaceticus* were 1.0091 ± 0.0003 and 1.0099 ± 0.0014, respectively. These values are slightly higher compared to $AKIE_c$ values previously gained for ring monohydroxylation of benzene (1.005 ± 0.0006) (Fischer et al. 2008) and toluene (1.008 ± 0.0002) (Morasch et al. 2002) by *Ralstonia picketti* strain PKO1 containing a toluene 3-monooxygenase (Byrne et al. 1995). Hydroxylation of toluene by toluene 4-monooxygenase, which is structurally related to toluene 3-monooxygenase (Leahy *et al.* 2003), is thought to proceed by interim formation of a position-specific epoxide (Mitchell et al. 2003). Notably, the $AKIE_c$ values obtained for the phenol hydroxylase of *A. calcoaceticus* are in the range of KIE_C values typical for a formation of a C=C bond epoxide as intermediate (1.00-1.01) and slightly smaller than KIE_c values typical for a C-H bond cleavage (1.0101-1.0204) (Elsner *et al.* 2005, Hunkeler and Elsner 2009) . However, further studies, e.g., by determining hydrogen isotope effects, are needed to give more solid conclusions regarding the reaction mechanism of phenol hydroxylase.

4.1.2 Isotope fractionation of methyl group hydroxylation of *p*-cresol

The degradation of *p*-cresol is initiated by a hydroxylation of the methyl group catalyzed by *p*-cresol methyl hydroxylase (PCMH). PCMH belongs to the flavin cytochrome *c* family and oxidize *p*-cresol to 4-hydrobenzyl alcohol while reducing flavin adenine dinucleotide (FAD) to $FADH_2$ (Cunane et al. 2000). Notably, the oxygen incorporated at the methyl moiety during the hydroxylation reaction catalyzed by PCMH stems from water and not from molecular oxygen. Correspondingly, PCMHs have been detected in facultative and strictly anaerobic bacteria, being active under oxic or anoxic conditions (Schink 2005, Fuchs *et al.* 2011). The oxidation of *p*-cresol to *p*-hydroxybenzyl alcohol by *Pseudomonas putida* NCIB 9866 proceeds by the utilization of one oxygen atom derived from water and the production of one reduced FAD (Hopper 1978, Cunane *et al.* 2000). Upon the degradation of *p*-cresol by *P. pseudoalcaligenes*, the AKIEc values gained (1.0164 ± 0.0014) indicate that hydrogen is abstracted from the carbon atom of the methyl moiety in the initial reaction when compared with typical KIEc values for a C-H bond cleavage (1.0101-1.0204) (Elsner *et al.* 2005). Also the denitrifying bacterium *A.buckelii* (designated earlier as *Paracoccus sp.* U120) was shown to initiate *p*-cresol degradation by methyl group hydroxylation catalyzed by PCMH (Hopper 1978, Rudolphi et al. 1991). The reaction takes place via H-abstraction and formation of a flavin radical,

which has been detected during the transition state (McIntire *et al.* 1985). Enrichment factors of -2.0 ± 0.1‰ and AKIEc values 1.0142 ± 0.001 were determined for *p*-cresol degradation by *A. buckelii*. The degradation of *p*-cresol by the strict anaerobe *G. metallireducens* was demonstrated to be initiated by methyl hydroxylation, too (Peters et al. 2007). However, the enrichment factor, ε_c=-3.6 ± 0.4‰ and AKIEc value 1.0259 ± 0.0027 gained were significantly higher than the values observed for *P. pseudoalcaligenes* and *A. buckelii*. A plausible reason is the obvious different PCMH structure and location in *G. metallireducens* than those in the other two strains (Peters *et al.* 2007), probably leading to different reaction mechanisms or transition states upon methyl group hydroxylation, eventually resulting in significant different $AKIE_C$ values.

In previous studies, the isotope fractionation due to the hydroxylation of the methyl moiety of toluene by *Pseudomonas putida* mt-2 containing a methyl monooxygenase was investigated, an enzymatic reaction which is formally similar to methyl group hydroxylation by PCMH including a C-H bond cleavage, but in which molecular oxygen is used as substrate instead of water. Notably, the AKIEc values for toluene oxidation by *P. putida* mt-2 (1.024 ± 0.002 (Morasch *et al.* 2002); 1.013 ± 0.001 (Mancini et al. 2006); 1.02 ± 0.002 (Vogt et al. 2008)) were in the same range as observed in this study for cresol hydroxylation by PCMH, indicating the C-H bond cleavage is the rate determining step in both reaction mechanisms. The result is also in line with the conclusion acquired from studies of aerobic toluene hydroxylation, that is, degradation initiated by methyl hydroxylation yields higher $AKIE_c$ values than the ring hydroxylation (Hunkeler and Elsner 2009) .

In addition, anaerobic side chain monohydroxylation was studied in *Aromatoleum aromaticum* EbN1 and *Georgfuchsia toluolica* G5G6 upon ethylbenzene degradation under nitrate reducing conditions by Dorer al. (2014b). The $AKIE_c$ values reported (1.030 ± 0.001, 1.033 ± 0.003) were closer to the data determined for *G. metallireducens* in this study. However, the ethylbenzene dehydrogenase is a molybdo-enzyme while PCMH is a flavocytochrome c, thus the reaction mechanisms of *p*-cresol and ethylbenzene oxidation are expected to be different (Peters *et al.* 2007, Dorer *et al.* 2014). Nevertheless, for both reactions, similar $AKIE_c$ of high magnitude were observed, indicating that the isotope sensitive C-H bond cleavage is the rate-determining step in each reaction.

4.1.3 Isotope fractionation of anaerobic phenol carboxylation

Carboxylation, the addition of CO_2 (usually in the form of bicarbonate) to an organic compound, has been proved to be an important strategy for anaerobic activation of non-substituted aromatic hydrocarbons, *n*-alkanes as well as phenols (Boll and Fuchs 2005, Mouttaki et al. 2012, Ji et al. 2013). Carboxylation of phenol by *T. aromatica* has been proposed to process through two major steps: the initial step is a reversible phosphorylation of phenol to phenylphosphate catalyzed by phenylphosphate synthase. This is followed by the irreversible intermediary dephosphorylation of phenylphosphate to a phenolate anion. Afterwards, the enzyme-bound phenolate reacts with the electrophilic carbon of carbon dioxide which leads to the carboxylation of the intermediate and formation of 4-hydroxybenzoate. (Schmeling et al. 2004, Schuhle and Fuchs 2004, Schmeling and Fuchs 2009). According to this reaction mechanism, no carbon atom is directly involved in the process of phenylphosphate formation, thus a primary kinetic isotope effect is not expected. Therefore, the small enrichment factors obtained from the studied carboxylation reaction by *T. aromatica* (ε_c = -0.7 ± 0.13‰) might be due to secondary isotope effects. Notably, a small but invers enrichment factor was generated from phenol degradation by *D. cetonica* (ε_c = 0.4 ± 0.08‰). The phenol degradation pathway of *D. cetonica* has not been confirmed yet; however, a putative phenylphosphate synthase gene and 4-hydroxybenzoate as a metabolite have been detected in a phenol-degrading sulfate-reducing bacterium (Ahn et al. 2009), indicating that phenylphosphate-dependent carboxylation is used by sulfate reducers, too.

4.1.4 Isotope fractionation of fumarate addition to activate cresols

Fumarate addition is a central strategy of hydrocarbon activation by anaerobes. These reactions are catalyzed by succinate synthase enzymes which are members of the glycyl radical enzyme family (Selmer et al. 2005). The degradation of *m*-cresol and *p*-cresol by the sulfate-reducer *D. cetonica* was also demonstrated to proceed via the fumarate addition mechanism (Müller *et al.* 1999, Müller *et al.* 2001). Toluene activation by fumarate addition under different redox conditions was shown to be associated with a significant carbon and hydrogen stable isotope fractionation. The enrichment factors for carbon stable isotopes ranged from -0.5‰ to -6.2‰, and the corresponding AKIEc values from 1.004 to 1.044 ± 0.008 while the $\varepsilon_{bulk,\ H}$ ranged from -23‰ to -98.4‰, corresponding to $AKIE_H$ values from 1.2255 to 4.6992 (Ahad et al. 2000, Ward et al.

2000, Tobler et al. 2008, Vogt *et al.* 2008). Isoenzymes of benzyl succinate synthase were reported to produce distinct carbon-hydrogen isotope pattern, allowing differentiating sulfate-reducing and nitrate-reducing toluene degraders (Vogt *et al.* 2008, Kümmel et al. 2013). Corresponding to these results, also *p*-cresol and *m*-cresol activation by fumarate addition was associated with moderate carbon isotope fractionation. In addition, the calculated $AKIE_c$ values were in the range of the typical KIEs for a cleavage of C-H bond (Elsner *et al.* 2005). Notably, the carbon enrichment factor for *m*-cresol degradation of *D. cetonica* observed in our study (-2.2 ± 0.3‰) differed significantly from the value (-3.9‰) reported by Morasch and colleagues (Morasch et al. 2004); in contrast, the values for *p*-cresol degradation were similar (-1.6‰ and -1.9 ± 0.5‰, respectively). The different results for *m*-cresol degradation could be due to the different analytical methods employed for carbon stable isotope measurement; in the previous study, cresols carbon fractionation was analyzed via GC-IRMS (Morasch *et al.* 2004). In our laboratory, we observed in numerous tests that cresols and phenol carbon isotope analysis by GC-IRMS is significantly affected by instrument-specific isotope fractionation (data not shown). The degradation of *m*-cresol by *D.cetonica* produced enrichment factors of -96.4 ± 49.1‰ for hydrogen stable isotope and $AKIE_H$ values of 3.1 ± 1.5. This is in accordance with the $\varepsilon_{bulk,\ H\ and}$ $AKIE_H$ reported for the same mechanism. For example, the hydrogen isotope fractionation upon toluene degradation by *D.cetonica* was studied by Vogt and colleagues(Vogt *et al.* 2008), and the bulk enrichment factor for hydrogen stable isotope was reported as -68 ‰, -74 ‰ and -80 ‰. However, due to the high error of the data, a repetition of the hydrogen isotope fractionation upon the biodegradation of *m*-cresol by *D.cetonia* is important and beneficial.

4.1.5 General conclusions and environmental implications

In summary, the constraints in existing analytical methods for phenols isotope fractionation analysis were overcome and a validated method was developed. Subsequently, the first data set of carbon enrichment factors and corresponding AKIEs upon the biodegradation of phenol and cresols by different activation mechanisms was acquired, providing the basis for an understanding of certain reaction mechanisms from the isotopic perspective. Furthermore, our data indicate that environmental monitoring of phenol and cresols biodegradation by compound specific carbon isotope analysis is principally possible due to the moderate to strong carbon isotope effects associated with

the aerobic and anaerobic activation steps of the major phenol and cresols degradation pathways. As an exception, detecting anaerobic phenol biodegradation is probably not possible by carbon isotope analysis as the initial reaction of the only known degradation pathway so far, the ATP-driven synthesis of phenylphosphate by phenylphosphate synthase, is not associated with the cleavage of a carbon bond, hence resulting in only minor carbon isotope fractionation . Notably, the reaction is linked to the breakage of an O-H bond and the formation of an O-P bond which could be associated to a primary hydrogen or oxygen isotope effect. However, the formation of phenylphosphate is reversible and not the rate-limiting step of the whole reaction sequence (Schmeling *et al.* 2004) and thus probably not isotope-sensitive.

The next step would be to test these assumptions by compound-specific carbon isotope analysis at a phenol/cresols contaminated site. The robustness of CSIA for environmental detection of pollutant biodegradation has been previously shown in several field studies (Sherwood Lollar et al. 2001, Song et al. 2002, Richnow et al. 2003, Richnow et al. 2003, Kaschl et al. 2005, Fischer et al. 2009). Furthermore, carbon isotope analysis combined with hydrogen isotope analysis (two-dimensional compound specific isotope analysis) would strongly increase the validity of the isotope approach. The hydrogen isotope analysis by combining the acylation of phenols prior to the isotope determination and the application of the new method with GC-Cr/HTC-IRMS has been successfully established for further work of this project. Summarized, the present work indicates a strong potential of using stable isotope tools to monitor phenols biodegradation in the environment.

4.2 Characterization of carbon isotope fractionation upon the anaerobic biodegradation of *n*-hexadecane by *D.oleovorans* strain Hxd3

The inert chemical properties, low water solubility and the tendency of accumulation in cell membranes make the utilization of *n*-alkanes by microorganisms difficult (Callaghan *et al.* 2006). Under anoxic condition, without the assistance of oxygen, the activation is of *n*-alkanes is even more demanding. Till now, several sulfate-reducers and nitrate-reducers have been reported as anaerobic *n*-alkane degraders. (Aeckersberg *et al.* 1991, Rueter *et al.* 1994, Aeckersberg et al. 1998, So and Young 1999, So and Young 1999b, Ehrenreich *et al.* 2000, Bonin et al. 2004, Cravo-Laureau et al. 2004). Three degradation pathways have been proposed for anaerobic *n*-alkane degradation: fumarate addition (So and Young

1999), hypothesized carboxylation(So and Young 1999) and hypothesized hydroxylation (Heider *et al.* 2016).

The sulfate-reducing *Desulfococcus oleovorans* strain Hxd3 is the first strain proved to be able to degrade *n*-alkanes under anaerobic condition (Aeckersberg *et al.* 1991). The strain has been reported to grow on *n*-alkanes with chain length from C_{12} to C_{20} as well as on 1-hexadecene, 1-hexadecanol, 2-hexadecanol, palmitate and stearate (Aeckersberg *et al.* 1991).

However, the genes coding for the enzymes involved in the fumarate addition pathways were not detected in the genomes of Hxd3 (Aeckersberg *et al.* 1998, So *et al.* 2003), indicating that the bacterium degrades *n*-alkanes by a different pathway.

By using ^{2}H and ^{13}C-labelled substrates, So and colleagues (So *et al.* 2003) described that the cellular lipids composition of strain Hxd3 was dependent on the chain lengths of the alkane substrates. C-odd alkanes were transformed to C-even fatty acids and C-even alkanes were transformed to C-odd fatty acids. When the strain was cultivated using unlabeled *n*-hexadecane as substrate and with the addition of ^{13}C labeled bicarbonate, the producted 15:0 fatty acid incorporated a ^{13}C label into the carboxyl group. In further experiments in which the strain was cultivated on 1,2-^{13}C labeled *n*-hexadecane, two types of 15: 0 fatty acids were detected: one with two-labeled ^{13}C at one chain-terminal, the other with no labeled carbon atom at all. So and colleagues proposed therefore the initial attack happened at C-3 position of the *n*-hexadecane(So *et al.* 2003). Moreover, by using ^{13}C-labelled bicarbonate in the growth medium, it was revealed the initial reaction was possibly carboxylation: a carboxyl group was added to the C-3 position of the *n*-hexadecane (So *et al.* 2003).

Interestingly, the hypothesized intermediate of *n*-hexandecane upon the carboxylation pathway, 2-ethylpentadecanoic acid, was never detected in studies that support the carboxylation pathway (So and Young 1999b, So *et al.* 2003, Callaghan *et al.* 2006, Callaghan *et al.* 2009). On the other hand, genome analysis showed the absence of genes encoding an alkane-activating glycyl radical enzyme and the presence of EbDH-like gene complex in strain Hxd3 (Callaghan 2008). Based on the above described results, Callaghan and colleagues described that the *n*-hexadecane might be activated by

hydroxylation at the C2 and the produced secondary alcohol is further oxidized to a ketone similar to the production of acetophenone is produced during ethylbenzene degradation (Callaghan 2013). Subsequently the ketone may possibly be carboxylized at C3 and a fatty acid that is one carbon shorter than the alkane is generated (Callaghan 2013). This hypothesis is consistent with all the studies so far (So *et al.* 2003, Heider and Schühle 2013, Heider *et al.* 2016).

Heider and colleagues proposed also the hydroxylation activation mechanism upon the degradation of *n*-alkanes by strain Hxd3 based on metabolites, lipids, genome and proteomics analysis. They confirmed that no genes coding the fumarate-adding enzyme is present, instead, genes coding for enzyme assembles the EbDH are present in the genome of strain Hxd3 (Heider and Schühle 2013). Furthermore, they found out that strain Hxd3 was able to grow on hexadecane-2-ol, which is an intermediate upon the hypothesized hydroxylation pathway. By analyzing the substrate-induced proteins, an EbDH-like molybdo-enzyme was found to be expressed in the cells grown on hexadecane but not in cells grown on hexadecanol or palmitate. Together with the further growth substrate and cellular lipids analysis, it was concluded that a hydroxylation catalyzed by an EbDH-like enzyme at the C-2 position was the initial step of *n*-alkanes degradation by *D. oleovorans* strain Hxd3 (Sünwoldt et al. unpublished results, personal communication).

The complete degradation of 1 mM *n*-hexadecane by *D.oleovorans* strain Hxd3 culture took approximately 3 months which is in accordance with studies before and the lab experience from which the culture was obtained (Aeckersberg *et al.* 1998, So *et al.* 2003). Two cultivation methods were tested in the experiments, one with the direct addition of *n*-hexadecane to the cultures and the other groups of culture using *n*-hexane as a carrier phase for *n*-hexadecane. *n*-Hexane was chosen as the carrier-phase for the following reasons: the stain Hxd3 can only degrade alkanes with chain-length over C_{12} (Aeckersberg *et al.* 1991), therefore, *n*-hexane is not degradable by the bacterium; 2) a carrier phase can insure an evenly distribution of the substrate during the cultivation time and is a commonly used method in bacterial cultivation with low-solubility substrates (Kühner et al. 2005, Kümmel et al. 2016); 3) the carrier phase can be directly taken into the subsequent concentration and isotope analysis. However, the growth of cultures grown with a carrier phase stopped after 20-40 days. The plausible reason for the phenomenon might be the higher solubility of *n*-hexane in water. It led to possible

accumulation of *n*-hexane in the cells and alteration of the cell membrane components (Fischer et al. 1982, Kim et al. 2002, Labinger and Bercaw 2002). Therefore, *n*-hexane is proved to be not a suitable carrier phase for this experiment. With another cultivation method, in which the *n*-hexadecane was directly injected into the culture, the culture grew.

Enrichment in the ^{13}C in *n*-hexadecane was not observed in the fractionation experiment. An insignificant enrichment factor of -0.1 ± 0.098 (AKIEc= 1.0016 ± 0.0016) was obtained. The stable isotope fractionation during anaerobic degradation of *n*-alkanes has been most extensively studied for propane and *n*-butane. Kniemeyer and colleagues (Kniemeyer *et al.* 2007) reported firstly the isotope fractionation of propane and *n*-butane degradation by several strains with the mechanism of fumarate-addition. For propane degradation, strain BuS5, Prop12-GMe and Propane60-GuB gave the $\varepsilon_{bulk\ C}$ (‰) of -5.2, -5.9 and -5.9 accordingly. Upon the degradation of *n*-butane, $\varepsilon_{bulk\ C}$ (‰) of -1.6 was observed for both the strain BuS5 and But12-GMe (Kniemeyer *et al.* 2007). Jaekel and colleagues reported isotope fractionation upon propane and *n*-butane degradation for the same strains, which were different from the study described above (Table 6.1) (Jaekel *et al.* 2014). They observed that under different cultivation condition, either with continuous shaking or static, the isotope fractionation values obtained were different (Jaekel *et al.* 2014). Mastalerz and colleagues studied propane and *n*-butane degradation by an anaerobic marine sediments enrichment culture with unknown degradation mechanism and obtained $\varepsilon_{bulk\ C}$ (‰) of –4.8 ± 3.1, $\varepsilon_{bulk\ H}$ (‰) of -43.3 ± 5.1 upon propane degradation and –0.7 ± 0.49 upon *n*-butane degradation (Mastalerz et al. 2009). Kinnaman and colleagues determined the carbon and hydrogen isotope fractionation factors associated with the aerobic degradation of methane to butane. They observed a decrease of carbon and hydrogen enrichment factor with the increasing of chain length (Kinnaman *et al.* 2007). Bouchard and colleagues studied the aerobic degradation of *n*-alkane with chain length up to 10 by soil microcosms. They observed also a decrease of carbon enrichment factor with the increasing of chain length. Isotope fractionation of *n*-decane was almost insignificant (Table 4.1), indicating that strong isotope masking might have taken place. They hypothesized that transport and binding steps of the *n*-alkane as substrate become more rate-limiting with the increasing of chain length (Bouchard *et al.* 2008).

This insignificant fractionation ($\varepsilon_{bulk,c}$= -0.1 ± 0.098, AKIEc= 1.0016 ± 0.0016) might not support the hypothesis of an initial step of hydroxylation catalyzed by an EbDH-like

enzyme. The EbDH catalyzes the initial step in the anaerobic ethylbenzene degradation by nitrate-reducers which is an oxygen-independent oxidation of ethylbenzene to 1-phenylethanol. It can also catalyze the hydroxylation of various alkylaromatic and heterocyclic compounds to the substituted alcohols (Knack et al. 2012). The enzyme and the ethylbenzene-metabolism mechanism was most well studies in the nitrate-reducer *Azoarcus* strain EbN1 (Kniemeyer and Heider 2001). The EbDH is a molybdenum enzyme belongs to the family of dimethyl sulfoxide reductase with three subunits. It contains four iron-sulfur clusters and a Mo and a heme b cofactor (Kloer et al. 2006). The hydroxylation of ethylbenzene was coupled to the reduction of the cofactor Mo (VI) to the Mo (IV). The mechanism was proposed to be a proton extraction by the Mo-cofactor to form a transient intermediate to reacts with the hydroxyl group stemming from H_2O to form the 1-phenylethanol. The reoxidation of the Mo (IV) was achieved by the electron transfer via iron-sulfur clusters and heme b to the electron acceptor (Szaleniec et al. 2009). The hydroxylation involves in a proton-extraction/ H-abstraction step which included a C-H bond cleavage. Therefore, a significant carbon isotope fractionation enrichment factor is expected for this type of reaction (Hunkeler and Elsner 2009). The bulk enrichment factor upon ethylbenzene degradation by the strain EbN1 has been reported as -3.8 ± 0.1‰ by Dorer and colleagues (Dorer *et al.* 2014).

The insignificant fractionation obtained might point to a possibility of carboxylation. Carboxylation has been proved to be an important strategy for anaerobic activation of non-substituted aromatic hydrocarbons and phenols (Boll and Fuchs 2005, Mouttaki *et al.* 2012). As described in chapter 3.1.3, the carboxylation of phenol by *T. aromatica* has been proposed to process through two major steps: phosphorylation and carboxylation (Schmeling *et al.* 2004, Schuhle and Fuchs 2004, Schmeling and Fuchs 2009). A secondary isotope effect is expected for the initial phosphorylation reaction which corresponds to a small AKIEc value. The same mechanism is reported also for acetone carboxylase. Upon the degradation of ethylbenzene, the intermediate acetophenone is

Table 4.1 Isotope fractionation factors for different activation mechanisms upon *n*-alkane degradation

Strain/Culture	Substrate	Activation Mechanism	ε_{bulkC}	AKIEc	ε_{bulkH}	$AKIE_H$	Reference
Strain BuS5	Propane, sulfate-reducing	Fumarate Addition	-5.2	1.016	n.d.	n.d.	Kniemeyer et al, 2007
Strain BuS5	Propane, sulfate-reducing	Fumarate Addition	-2.6 ± 0.6	1.008 ± 0.002	-16 ± 4	1.138 ± 0.041	Jaekel et al., 2014
Strain BuS5	Propane, sulfate-reducing	Fumarate Addition	-8.7 ± 1.1	1.026 ± 0.003	-92 ± 6	2.143 ± 0.286	Jaekel et al., 2014
Prop12-GMe	Propane, sulfate-reducing	Fumarate Addition	-5.9	1.018	n.d.	n.d.	Kniemeyer et al, 2007
Prop12-GMe	Propane, sulfate-reducing	Fumarate Addition	-3.1 ± 0.4	1.009 ± 0.001	-24 ± 4	1.217 ± 0.041	Jaekel et al., 2014
Prop12-GMe	Propane, sulfate-reducing	Fumarate Addition	-3.7 ± 0.5	1.011±0.002	-36 ± 4	1.352 ± 0.065	Jaekel et al., 2014
Propane60-GuB	Propane, sulfate-reducing	Fumarate Addition	-5.9	1.018	n.d.	n.d.	Kniemeyer et al, 2007
Marine Sediments	Propane, sulfate-reducing	Unknown	-4.8 ± 3.1	n.d.	-43.3 ± 5.1	n.d.	Mastalerz et al., 2009
Strain BuS5	*n*-Butane, sulfate-reducing	Fumarate Addition	-1.6	1.006	n.d.	n.d.	Kniemeyer et al, 2007
Strain BuS5	*n*-Butane, sulfate-reducing	Fumarate Addition	-1.8 ± 0.3	1.007 ± 0.001	-9 ± 2	1.093 ± 0.026	Jaekel et al, 2014

Strain BuS5	*n*-Butane, sulfate-reducing	Fumarate Addition	-5.0 ± 0.7	1.02 ± 0.003	-32 ± 10	1.423 ± 0.196	Jaekel et al, 2014
But12-GMe	*n*-Butane, sulfate-reducing	Fumarate Addition	-1.6	1.006	n.d.	n.d.	Kniemeyer et al, 2014
But12-GMe	*n*-Butane, sulfate-reducing	Fumarate Addition	-2.0 ± 0.5	1.008 ± 0.002	-11 ± 4	1.127 ± 0.048	Jaekel et al, 2014
But12-GMe	*n*-Butane, sulfate-reducing	Fumarate Addition	-5.6 ± 0.5	1.023 ± 0.002	-47 ± 5	1.780 ± 0.135	Jaekel et al, 2014
But12-HyR[a]	*n*-Butane, sulfate-reducing	Fumarate Addition	-0.8 ± 0.3	1.003 ± 0.001	-5 ± 2	1.050 ± 0.023	Jaekel et al, 2014
But12-HyR[a]	*n*-Butane, sulfate-reducing	Fumarate Addition	-1.5 ± 0.4	1.006 ± 0.001	-12 ± 2	1.129 ± 0.031	Jaekel et al, 2014
Marine Sediments	*n*-Butane, sulfate-reducing	Unknown	-0.7 ± 0.4	n.d.	n.d.	n.d.	Mastalerz et al. 2009
Soil microcosms	Propane, aerobic	Unknown	-10.8 ± 0.7	1.003	n.d.	n.d.	Bouchard et al. 2007
Soil microcosms	*n*-Butane, aerobic	Unknown	–5.6 ± 0.1	1.023	n.d.	n.d.	Bouchard et al. 2007
Soil microcosms	*n*-Pentane, aerobic	Unknown	–2.4 ± 0.2	1.034	n.d.	n.d.	Bouchard et al. 2007
Soil microcosms	*n*-Hexane, aerobic	Unknown	–2.3 ± 0.6	1.013	n.d.	n.d.	Bouchard et al. 2007
Strain HxN1	*n*-Hexane,	Unknown	n.d.	1.0023	n.d.	n.d.	Vieth and Wilkes 2006

	nitrate-reducing						
Oil field microcosms	*n*-Hexane, nitrate-reducing	Unknown	n.d.	1.0016	n.d.	n.d.	Vieth and Wilkes 2006
Soil microcosms	*n*-Octane, aerobic	Unknown	-0.9 ± 0.1	1.007	n.d.	n.d.	Bouchard et al. 2007
Soil microcosms	*n*-Decane, aerobic	Unknown	-0.2 ± 0.1	1.002	n.d.	n.d.	Bouchard et al. 2007
Marine Sediments	ethane, aerobic	Unknown	-10.1 ± 1.0	n.d.	-156.4	n.d.	Kinnaman et al. 2007
Marine Sediments	ethane, aerobic	Unknown	-8.5 ± 0.4	n.d.	-319.9	n.d.	Kinnaman et al. 2007
Marine Sediments	*n*-propane, aerobic	Unknown	-4.3 ± 1.5	n.d.	-13.8	n.d.	Kinnaman et al. 2007
Marine Sediments	*n*-propane, aerobic	Unknown	-5.4 ± 0.2	n.d.	-16.5	n.d.	Kinnaman et al. 2007
Marine Sediments	*n*-butane, aerobic	Unknown	-2.4 ± 1.0	n.d.	n.d.	n.d.	Kinnaman et al. 2007
Marine Sediments	*n*-butane	Unknown	-3.4	n.d.	n.d.	n.d.	Kinnaman et al. 2007

n.d.: not determined

carboxylated in the same way to benzoylacetate (Kniemeyer and Heider 2001). It is also proposed that in the alkane-degrading *D.oleovorans* strain Hxd3, after the hydroxylation of *n*-hexadecane at C2, the produced secondary alcohol is further oxidized to a ketone in the way which acetophenone is produced during ethylbenzene degradation (Callaghan 2013, Heider *et al.* 2016). Another variant of carboxylation has been reported for the anaerobic degradation of naphthalene. In the initial step of the degradation, naphthalene was carboxylized to 2-carboxylnaphthoic acid catalyzed by naphthalene carboxylase (Meckenstock *et al.* 2000, Mouttaki *et al.* 2012). In the study, the sulfate-reducing naphthalene-degrading enrichment culture N47 was cultivated with ^{13}C-labelled bicarbonate. The production of ^{13}C-labelled 2-naphthoic acid was observed and this reaction is not dependent on ATP, thus indicating a direct carboxylation (Mouttaki et al. 2012). This reaction is also suggested for a naphthalene-degrading, sulfate-reducing marine strain NaphS2 and NaphS6 (Musat et al. 2009).This mechanism can possibly lead to larger carbon isotope fractionation than the first carboxylation variant. Bergmann and collegues studied the carbon and hydrogen isotope fractionation pattern by the naphthalene-degrading sulfate reducer NaphS2 and they obtained a moderate carbon and hydrogen isotope fractionation factor upon this hypothesized carboxylation activation mechanism ($\varepsilon_{bulk,c}$= -2 ± 0.4 ‰, AKIEc= 1.02; $\varepsilon_{bulk,H}$= -43 ± 6‰, $AKIE_H$= 1.41). In comparison, the culture N47 used in the same study with the same hypothesized carboxylation mechanism produced insignificant carbon fractionation and moderation hydrogen fractionation factor ($\varepsilon_{bulk,c}$= -0.3 ± 0.1‰, AKIEc= 1.003; $\varepsilon_{bulk,H}$= -59 ± 8‰, $AKIE_H$= 1.67) (Bergmann et al. 2011). The study by Kümmel and colleagues using strain NaphS6 obtained the similar result with the ones for culture N47 ($\varepsilon_{bulk,c}$= -0.4 ± 0.3‰; $\varepsilon_{bulk,H}$= -11± 2‰ to -47± 4 ‰ , $AKIE_H$= 1.1 to 1.57) (Kümmel *et al.* 2016). These studies suggest that the second type of carboxylation mechanism can but not always lead to a larger fractionation than the first type.

The masking of isotope fractionation can also lead to the insignificant isotope fractionation observed. The transport of *n*-alkanes into the cells and the binding step to the reactive site becomes more rate-limiting with the increase of chain length (Bouchard *et al.* 2008). It can mask part of the actual isotope fractionation caused by the initial reaction upon the biodegradation pathway (Bouchard *et al.* 2008). The study of Bouchard and collegues gave an example of the possible masking effect appeared with the biodegradation of *n*-alkanes. In their study, the degradation of *n*-alkanes with chain length

from C3 to C10 are all initiated by aerobic hydroxylation (Bouchard *et al.* 2008). Taking into consideration of the dilution effect with the increase of carbon atoms, the bulk fractionation factor of the reaction site is calculated and listed in table 6.2. Despite the exclude of dilution effect, the $\varepsilon_{\text{reactive position}}$ and AKIE still decrease with the increase in chain length. In comparison, in another study of carbon stable isotope fractionation upon chemical reaction of *n*-alkanes with OH radical in the gas phase, the AKIE did not show this pattern: it remains similar for all compounds (Table 4.2) (Anderson et al. 2004). This comparison provided evidence that during biodegradation of *n*-alkanes with longer chain, the transport and binding step are rate-limiting and thus masks the stable isotope fractionation produced by the initial reaction.

Table 4.2 Carbon stable isotope fractionation during aerobic hydroxylation and chemical transformation of *n*-alkanes*

		Biodegration			Chemical transformation
n-alkanes	chain length	ε_{bulk} (‰)	$\varepsilon_{\text{reactive postion}}$ (‰) #	$AKIE_{bio}$	$AKIE_{chemical}$
propane	3	-10.8	-16.5	1.033	1.014
n-Butane	4	-5.6	-11.2	1.022	1.013
n-Pentane	5	-3.8	-9.5	1.019	1.014
n-Hexane	6	-2.3	-6.9	1.013	1.021
n-Heptane	7	-1.4	-4.9	1.010	1.017
n-Octane	8	-0.9	-3.6	1.007	1.017
n-Decane	10	-0.2	-1.0	1.002	n.d.
n-Hexadecane	16	-0.1	-0.8	1.002	n.d.

* The data of biodegradaion of propane to *n*-decane comes from the study by Bouchard and collegues (Bouchard *et al.* 2008). The data of chemical transformation comes from the study by Anderson and collegues (Anderson *et al.* 2004). The data of *n*-hexadecane biodegradation comes from this study.

$\varepsilon_{\text{reactive position}} = \varepsilon_{bulk} \times$ number of carbon atoms in the molecule/number of carbons atoms at the reaction site

A new trial to analyze the hydrogen isotope fractionation would possibly give a more concrete evidence for the activation mechanism upon *n*-hexadecane degradation by *D.oleovorans* strain Hxd3.

4.3 The microbial communities in the anaerobic ethylbenzene-degrading, sulfate-reducing enrichment culture obtained from a contaminated aquifer in Zeitz, Germany

The enrichment was performed with ethylbenzene as electron donor and growth substrate and sulfate as the main electron acceptor. The accumulation of sulfide in the enrichment culture proved that the electrons stemming from ethylbenzene oxidation were coupled to sulfate reduction. The stoichiometry of the ethylbenzene oxidation coupled to sulfate reduction is as follows: $C_6H_5C_2H_5 + 5.25\ SO_4^{2-} + 3\ H_2O \rightarrow 8\ HCO_3^- + 5.25\ HS^- + 2.75\ H^+$ (Kniemeyer *et al.* 2003). It is however not possible to generate an accurate stoichiometry for the ethylbenzene-degrading, sulfate-reducing enrichment culture in this study. The first reason is the extreme slow mineralization rate of ethylbenzene, verified by the SIP experiment. Secondly, the lost amount of ethylbenzene in the abiotic control cultures was quite significant, thus making it difficult to calculate the amount of biodegraded ethylbenzene. Thirdly, as this is an enrichment culture originating from an aquifer sampling system, the oxidation of background substrates leading to sulfide production cannot be excluded while part of the sulfide formed might further react with metal ions such as Fe^{2+} to form precipitation which coated on the sand particles in the culture.

In the SIP experiment, the enrichment of $^{13}CO_2$ in the cultures grown on labelled ethylbenzene showed evidence of the mineralization of ethylbenzene to CO_2. However, the extremely slow mineralization rate, 67 µM $^{13}CO_2$ produced in 522 cultivation days corresponding to 11 µM ethylbenzene, indicated the extremely slow degradation and growth of bacteria under the given condition. The most possible reason for the extreme growth rate of the culture is the growth condition provided does not meet the optimum growth condition for the ethylbenzene-degrader(s). This might be due to the lack of specific nutrients, competition for nutrients among different microorganisms or even release of bacteriocins from other species (Vartoukian *et al.* 2010). For example, in the case of *Dehalococcoides ethenogenes* 195, the concentration of vitamin B12 plays a vital role in the growth rate of the cultures. The growth rate doubled when the concentration of vitamin B12 in the culture is increased from 0.001 mg/L to 0.025 mg/L (He et al. 2007). Köpke and colleagues studied the impact of cultivation condition on the microbial communities and isolates using coastal subsurface sediments. 112 cultures were isolated and each cultivation condition led to a specific isolates confirming the influence of cultivation approaches on the yield of culture (Köpke et al. 2005). Some bacteria have

been proven to enter into a temporary state of low metabolic activity when facing unfamiliar environment in the lack of essential factors (Barcina et al. 1990, Colwell 2000, Nichols et al. 2008).

Our attempts to grow an ethylbenzene-degrading aqueous culture without sand have failed so far. In other approaches, yeast extracts have been additionally amended to assist the growth of microorganisms (Gaudreau et al. 1999, Gorret et al. 2001). In this thesis, yeast extracts have been added to several enrichment cultures. However, a significant increase in sulfide concentration was observed without obvious acceleration in the ethylbenzene degradation rate (data not shown).

Cultivation with dilute nutrient media (Watve et al. 2000, Connon and Giovannoni 2002, Zengler et al. 2002), the addition of signaling molecules such as cyclic AMP (Bruns et al. 2002) and mimicking the natural environment (Kaeberlein et al. 2002, Bollmann et al. 2007, Song et al. 2009, Wang et al. 2009) have been confirmed as useful techniques in the enrichment of environmental microorganisms. These approaches are worthy of testing in the further enrichment of the Zeitz ethylbenzene-degrading culture.

The identified proteins in the metaproteomics analysis were mainly assigned to the phylum of Firmicutes and Proteobacteria. Clostridiales is the dominating orders of Firmicutes in this case and Deltaproteobacteria were mainly composed of the orders Desulfobacterales, Desulfuromonadales and Syntrophobacterales. The relative abundance of orders assigned to the most important functional groups indicated that Clostridiales and Archaeoglobales were the dominant orders for C1 metabolism/CO_2 fixation. Dissimilatory sulfate reduction was related with Clostridiales, Syntrophobacterales and unclassified bacteria. The aromatic compound degradation groups were mainly affiliated to the orders Clostridiales and Rhodobacterales (Alphaproteobacteria).Clostridiales was described in studies as benzene degrader which fermented benzene to acetate and hydrogen (Herrmann et al. 2010, Vogt et al. 2011, Kleinsteuber et al. 2012, Taubert *et al.* 2012). Syntrophobacterales are often found to be affiliated with dissimilatory sulfate reduction (Kleinsteuber et al. 2008). Archaeoglobales are found in the Zeitz benzene-degrading culture as acetate-utilizer (Starke *et al.* 2016). Rhodocyclales are often reported to involve in nitrogen transformation and aromatic compounds degradation (Loy et al. 2005, Hesselsoe et al. 2009) . However, the influence on protein distribution by horizontal transfer among different microorganisms cannot be

excluded in this case (Wiedenbeck and Cohan 2011, Taubert *et al.* 2012). The proteins which were identified belonging to the functional group "aromatic compound degradation" are all related to the anaerobic degradation pathway of benzoate. This is probably the lower pathway of anaerobic ethylbenzene degradation (Kniemeyer *et al.* 2003, Rabus 2005) . No proteins are identified who are involved in ethylbenzene activation. The three key enzymes involved in the dissimilatory sulfate reduction: adenylylsulfate reductase, dissimilatory sulfide reductase and sulfate adenylyltransferase are all identified (Lee and Peck 1971, Segel et al. 1987). Enzymes identified which related to the Wood-Ljungdahl pathway CO dehydrogenase, acetyl-CoA synthase, methylenetetrahydrofolate dehydrogenase, methylenetetrahydrofolate reductase. Over 80% of these proteins are assigned to the Clostridiales (Ljungdahl 1986, Diekert and Wohlfarth 1994, Rabus et al. 2006).

The community structure revealed by the metaproteome and SIP experiments analysis shared high similarity with the one described for the benzene-degrading, sulfate-reducing culture enriched from Zeitz active sand material (Taubert *et al.* 2012, Starke *et al.* 2016). Taubert and colleagues described a benzene-degrading, sulfate-reducing enrichment culture that was cultivated with ^{13}C-labeled benzene and ^{13}C-labeled carbonate. The subsequent functional groups analysis and assignment revealed that the benzene-degrading, sulfate-reducing process was a syntrophic reaction. The benzene degradation was initiated by the Clostridiales, they also played the significant role in CO_2 fixation. A group of Deltaproteobacteria used the metabolites from benzene-degrading process and reduced the sulfate. And the phyla Bacteroidetes and Chlorobis were proposed as a hypothetical scavenger group (Taubert *et al.* 2012). Starke and colleagues further verified and completed this hypothesis by metaproteomic analysis of the culture and functional groups analysis of the benzene-degrading, sulfate-reducing cultures grown with additional ^{13}C-labeled acetate as well as cultivation with elemental sulfur as electron acceptor. The addition of ^{13}C-acetate did not lead to an increase in sulfide production. A putative sulfide oxidation process by the Epsilonproteobacteria Campylobacterales was proposed also in this study and study by Keller and colleagues (Keller et al. 2015). The completed community structure in the benzene-degrading, sulfate-reducing culture was then proposed as contained benzene fermenter Clostridiales, the benzene metabolites utilizers Archaeoglobales, Campylobacterales, Syntrophobacterales, Clostriadiales and Desulfobacterales as well as the dead biomass scavenger Bacteroidetes, Chlorobi and

Chloroflexi (Herrmann *et al.* 2008, Vogt *et al.* 2011, Kleinsteuber *et al.* 2012, Taubert *et al.* 2012).

The groundwater in the Zeitz plant contained 300 µM benzene, <1 µM ethylbenzene and xylenes, 4 mM sulfate (Vogt *et al.* 2007). The original microbial communities in Zeitz column systemsis therefore adapted to the high benzene concentration. The extreme slow mineralization rate indicates the failed enrichment of the ethylbenzene-degraders in the culture. Hence, the the community structure was not that much shifted from the benzene-degrading cultures.

The metaproteome data differed considerably to the metagenome data (Figure 3.24, figure 3.31). Proteobacteria occupied the similar portion in both analyses. The result of metagenomic analysis showed a dominate abundance of the phylum Chloroflexi and minor abundance of Firmicutes while the metaproteomic analysis gave obvious the opposite result. The phylum Chloroflexi was proposed to act as the scavenger in the Zeitz enrichment cultures (Taubert *et al.* 2012, Starke *et al.* 2016), hence, a dominant abundance of this phylum could be an indication of the low degrading-activity in the enrichment culture which was sacrificed for metagenomics analysis. The other possible reason which leads to the difference of the two analyses might be the methodology. The sequence databases selection has always been a major challenge in the bioinformatics post-processing of meta-omics (Teeling and Glöckner 2012, Tanca et al. 2016, Timmins-Schiffman et al. 2017). The genome sequencing data was annotated based on the RDP bacterial database while the metaproteome data was annotated on base of a metagenome dataset from Zeitz contaminated aquifer. The database-specific bias chosen for the annotation of metagenome data and metaproteome data could also lead to this observed difference (Teeling and Glöckner 2012, Tanca et al. 2013, Aguiar-Pulido et al. 2016, Tanca *et al.* 2016, Timmins-Schiffman *et al.* 2017). Especially for metaproteome data, the different database may cover different parts of the acquired metaproteome (May et al. 2016). For example, Schiffman and colleagues studied the influence of database selection on the metaproteom result. They annotated the same analysis result against four different databases from the NCBI database to time/site-specific metagenome database. It was found out that the peptides identified by the four databases did not overlap much (Timmins-Schiffman *et al.* 2017).

4.4 Insight into the BTEX biodegradation in the field site of Òdena

The field study in Òdena within this thesis provided the first comprehensive isotopic composition of BTEX contaminants in different wells along the groundwater flow and at different depth level in each well of this site.

4.4.1 BTEX concentration and isotope composition

Well S3, which located upstream of the contaminant plume (Figure 3.3), exhibited significant higher concentration of BTEX compared to well S6 and S9. The concentration in well S6 is higher than in well S9. In well S9, the BTEX contaminants were barely detected. This demonstrated that a natural attenuation process of BTEX took place along the groundwater flow line. This distribution pattern of other contaminants at this site was also observed (Palau *et al.* 2014). For the evaluation of biodegradation at a contaminated site, isotope fractionation due to other processes should be insignificant. In most cases, the fractionation caused by biodegradation is significantly larger than by physical processes (Jeannottat and Hunkeler 2013, Imfeld et al. 2014, Wanner and Hunkeler 2015). When CSIA was applied as the tool to assess the biodegradation of organic pollutants, the US Environmental Protection Agency suggests that the differences between δ-values should be at least 4 times larger than the analytical uncertainty (± 0.5‰ for $\delta^{13}C$ values and ± 5‰ for $\delta^{2}H$ values) (Hunkeler et al. 2008). According to this principle, Fischer and colleagues recommends the following criteria for the assessment biodegradation by carbon stable isotope ratios: 1) for a certain organic pollutant, if a secondary contaminant source with a more positive $\delta^{13}C$ value is not present, a change of $\delta^{13}C$ value by larger than 2‰ along the groundwater flow path indicates the degradation of pollutant. A $\delta^{13}C$ value change between 1‰ and 2‰ points to a possible biodegradation of pollutant. 2) A $\delta^{13}C$ value more positive than -20‰ can be caused by biodegradation as most primary carbon isotope signatures are more negative than -22‰ (Thullner et al. 2012, Fischer et al. 2016). Similarly, the criteria for assessment biodegradation by hydrogen stable isotope ratios can be concluded as: 1) for a certain organic pollutant, if a secondary contaminant source with a more positive $\delta^{2}H$ value is not present, a change of $\delta^{2}H$ value by larger than 20‰ along the groundwater flow path indicates the degradation of pollutant. A $\delta^{2}H$ value change between 10‰ and 20‰ points to a possible biodegradation of pollutant. 2) A $\delta^{2}H$ value more positive than -30‰ can be caused by biodegradation as most primary hydrogen isotope signatures are more negative than -30‰. Only minor shifts with regard

to $\delta^{13}C$ values and $\delta^{2}H$ values with depth were observed in both wells. In S3, the maximum change in $\delta^{13}C$/ $\delta^{2}H$ values at different depth is 1.62‰/9.41‰ for toluene, 1.37‰/16.68‰ for ethylbenzene and 1.87‰/7.6‰ for *m,p*-xylene. Based on the criteria given above, these data give hint to biodegradation of toluene, ethylbenzene and *m,p*-xylene in well S3. In S6, the maximum change in $\delta^{13}C$ values at different depth is 0.76‰ for toluene, 2.26‰ for ethylbenzene and 3.37‰ for *m,p*-xylene. Furthermore, at the depth level 10.5 m, 11 m and 17.5 m in well S6, the $\delta^{13}C$ values for *m,p*-xyene are positive than -20‰. These evidence indicate the possible biodegradation of ethylbenzene and *m,p*-xylene in well S6. More significant shifts of $\delta^{13}C$ values were observed, from well S3 to S6 along the flow path: 6.7‰ for toluene, 3.4‰ for ethylbenzene and 9.7‰ for *m,p*-xylene. These data strongly suggested biodegradation process of toluene, ethylbenzene and *m,p*-xylene along the groundwater flow. Due to the complexity of the site (Palau *et al.* 2014, Torrentó et al. 2014), the shift in $\delta^{13}C$ between the two wells along the flow direction could still not indicate which potential biodegradation processes and where were they taking place. Method development for detecting isotope composition of contaminants with trace concentration can allow for a more comprehensive and reliable assessment of in situ BTEX degradation in the contaminated zone. The HS-PTV method with lower detection limit showed the potential for isotope composition analysis of samples from Òdena field site with low BTEX concentration. Nevertheless, the trial in this thesis with the HS-PTV facility failed to build a valid method due to the shift in isotope values obtained with the change of BTEX standards concentration. Further optimization of the method will help to extend the possibilities of CSIA for this field samples.

4.4.2 The enrichment of ethylbenzene-degrading cultures and characterization of the degradation

No obvious enrichment of ethylbenzene-degraders under sulfate-reducing, nitrate-reducing or iron-reducing conditions were obtained with the sludge from well S3. In the enrichment experiment with sludge from well S6 and S9, ethylbenzene-degrading nitrate-reducers were present and could be cultivated and enriched in the lab. This indicated the difference of in-situ microbial communities in well S3 in comparison with the other two wells. Under sulfate-reducing and iron-reducing conditions, no obvious acceleration of degradation was observed in cultures established with sludge from well S6 and S9, either. The lack of sulfate-reducers and iron-reducers from the original microbial communities or

the lack of optimal cultivation condition in the enrichment experiments might be the reason for this. The genus *Azoarcus* was identified as the only degrader in the enrichment culture from well S6 under nitrate-reducing condition and the genus *Thauera* for the culture from well S9 under nitrate-reducing condition. The two closely related genus *Thauera* and *Azoarcus* (Rhodocyclales, Betaproteobacteria, Proteobacteria, Bacteria) are the major groups reported of nitrate-reducers capable of degrading aromatic compounds (Anders et al. 1995, Rabus and Widdel 1995, Zhou et al. 1995, Gallus et al. 1997, Springer et al. 1998, Häggblom and Young 1999, Harms et al. 1999, So and Young 1999, Song et al. 1999, Song et al. 2001, Mao et al. 2010, Fernández et al. 2014). There are species from *Thauera* reported which are capable of both aerobic and anaerobic aromatic compounds degradation (Scholten et al. 1999, Shinoda et al. 2004, Chen and Strous 2013). This is in accordance with the redox condition at well S9 which is aerobic/ nitrate-reducing. The bacteria which are capable of anaerobic degradation of one or two BTEX compounds were often found alternatively capable of other BTEX compounds as well, especially the genus *Azoarcus* and *Thauera* (Dolfing et al. 1990, Fries et al. 1994). This is verified also in this thesis. The microcosms set up with sludge from S9 showed fast degradation ability of toluene. These result indicated that Betaproteobacteria belonging to the genera *Azoarcus* and *Thauera* might play an important role in the anaerobic bioremediation of BTEX compounds in well S6 and S9 at the field site Òdena. Experiments with function gene markers and growth substrate tests are planned to allow for a further identification of the two isolated strains.

There are only a limited number of isolated ethylbenzene degraders reported: strains EbN1 (Rabus and Widdel 1995), EB1 (Ball *et al.* 1996), *Georgfuchsia toluolica* strain G5G6 (Weelink *et al.* 2009), PbN1 (Rabus and Widdel 1995) and EbS7 (Kniemeyer *et al.* 2003). The former four strains are denitrifying bacteria and the last one is sulfate-reducer. The reported initial reaction of the degradation pathway of nitrate-reducers is methyl-hydroxylation (Rabus and Widdel 1995, Ball *et al.* 1996, Weelink *et al.* 2009). This mechanism was most extensively studies in the strain EbN1. The detailed reaction mechanism was discussed in chapter 1.2. The rate-limiting step of this reaction is the formation of a radical intermediate, by which a C-H bond cleavage is involved (Szaleniec et al. 2012). Therefore, a significant carbon isotope fractionation is expected for this type of reaction. An enrichment of $\delta^{13}C$ values from -30.2 ‰ to -18.2 ‰ was observed and a corresponding bulk enrichment factor of -3.7 ± 0.4‰ was calculated (Figure 3.35). The

AKIEc values for S6 and S9 were 1.0322 ± 0.0034 and 1.0305 ± 0.0034, accordingly (Table 3.7). The AKIEc values are in the range of KIE_c values typical for a C-H bond cleavage (1.0101-1.0204) (Elsner *et al.* 2005, Hunkeler and Elsner 2009). Dorer et al. have analyzed the carbon isotope fractionation upon the ethylbenzene-degradation by two of these nitrate-reducers: for *Aromatoleum aromaticum* EbN1, a $\varepsilon_{bulk,c}$ value of -3.8 ± 0.1‰ and AKIEc value of 1.030 ± 0.001was obtained and for *Georgfuchsia toluolica* G5G6 a $\varepsilon_{bulk,c}$ value of -4.1 ± 0.2‰, AKIEc value of 1.033 ± 0.001 was gained (Dorer *et al.* 2014). These data was in the same rage with the values determined for the S6, S9 enrichment cultures. Hence, it is concluded that methyl group hydroxylation is the initial reaction upon the degradation of ethylbenzene pathway by the two cultures.

In summary, the study at the field site of Òdena provided the first insight into the BTEX degradation at this site. Stable isotope analysis provided hints for possible biodegradation of TEX in well S3 and possible biodegradation of ethylbenzene and *m,p*-xylene in well S6. Moreover, there is significant shift in $\delta^{13}C$ values of TEX compounds along the flow of groundwater which indicated a possible biodegradation process. The genus *Azoarcus* and *Thauera* might play the major role of BTEX removal from the wells S6 and S9, respectively. And under nitrate reducing condition, the activation mechanism of the ethylbenzene degradation of the S6 and S9 cultures are methyl group hydroxylation.

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Author's declaration of originality

I, Xi Wei, born on 04.02.1989 in China, declare that I am aware of the official doctoral regulations of the Faculty of Biological Sciences of the Friedrich Schiller University Jena. This thesis has been composed entirely by me. I have clearly referenced in both the text and the bibliography of references, all sources used in this work. No commercial activities are related to the contents of this work. This dissertation has not been published before. This dissertation has only been submitted to the Council of the Faculty of Biology and Pharmacy of the Friedrich Schiller University Jena, and not to any other university for academic examination or dissertation. This thesis is neither identical nor partially identical to any work which has been submitted as dissertation to the Friedrich Schiller University Jena or any other universities.

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Essen,_____

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02/2013 – 01/2018	PhD thesis *Helmholtz Center for Environmental Research, Leipzig, Germany, Department Isotope Biogeochemistry* Research focus: ▪ Development, optimization und validation of analytical methods of gas-chromatography, liquid-chromatography, mass-spectrometry and isotope-mass-spectrometry ▪ Cultivation and characterization of microorganisms ▪ Sampling in the field
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Publication records

Xi Wei, Tetyana Gilevska, Felix Wetzig, Conrad Dorer, Hans-Hermann Richnow, Carsten Vogt, Characterization of phenol and cresol biodegradation by compound-specific stable isotope analysis, Environmental Pollution, Volume 210, March 2016, Pages 166-173.

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2014 Enrichment and characterization of a freshwater sulfate-reducing ethylbenzene-degrading culture. **X.Wei**, C. Dorer, M. Duarte, D.H. Pieper, P. Bombach, H.H. Richnow, C. Vogt. **Poster presentation. Novel Anaerobe Symposium, Wagningen, Netherland.**

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